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(54) Title: METHODS AND COMPOSITIONS FOR STIMULATING BONE CELLS

(57) Abstract

Disclosed are methods, compositions, kits and devices for use in transferring nucleic acids into bone cells *in situ* and/or for stimulating bone progenitor cells. Type II collagen and, particularly, osteotropic genes, are shown to stimulate bone progenitor cells and to promote bone growth, repair and regeneration *in vivo*. Gene transfer protocols are disclosed for use in transferring various nucleic acid materials into bone, as may be used in treating various bone-related diseases and defects including fractures, osteoporosis, osteogenesis, imperfecta and in connection with bone implants.

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DESCRIPTION

Methods and Compositions for Stimulating Bone Cells

The present application is a continuation-in-part of U.S. Serial Number 08/316,650, filed September 30, 1994; which is a continuation-in-part of U.S. Serial Number 08/199,780, filed February 18, 1994; the entire text and figures of which disclosures are specifically incorporated herein by reference without disclaimer. The United States government has certain rights in the present invention pursuant to Grant HL-41926 from the National Institutes of Health.

1. Field of the Invention

The present invention relates generally to the field of bone cells and tissues. More particularly, certain embodiments concern the transfer of genetic material into bone and other embodiments concern type II collagen. In certain examples, the invention concerns the use of type II collagen and nucleic acids to stimulate bone growth, repair and regeneration. Methods, compositions, kits and devices are provided for transferring an osteotropic gene into bone progenitor cells, which is shown to stimulate progenitor cells and to promote increased bone formation *in vivo*.

2. Description of the Related Art

Defects in the process of bone repair and regeneration are linked to the development of several human diseases and disorders, *e.g.*, osteoporosis and osteogenesis imperfecta. Failure of the bone repair mechanism is, of course, also associated with significant complications in clinical orthopaedic practice, for example, fibrous non-union following bone fracture,

implant interface failures and large allograft failures. The lives of many individuals would be improved by the development of new therapies designed to stimulate and strengthen the fracture repair process.

Naturally, any new technique to stimulate bone repair would be a valuable tool in treating bone fractures. A significant portion of fractured bones are still treated by casting, allowing natural mechanisms to effect wound repair. Although there have been advances in fracture treatment in recent years, including improved devices, the development of new processes to stimulate, or complement, the wound repair mechanisms would represent significant progress in this area.

A very significant patient population that would benefit from new therapies designed to promote fracture repair, or even prevent or lessen fractures, are those patients suffering from osteoporosis. The term osteoporosis refers to a heterogeneous group of disorders characterized by decreased bone mass and fractures. Clinically, osteoporosis is segregated into type I and type II. Type I osteoporosis occurs predominantly in middle aged women and is associated with estrogen loss at the menopause, while osteoporosis type II is associated with advancing age.

An estimated 20-25 million people are at increased risk for fracture because of site-specific bone loss. The cost of treating osteoporosis in the United States is currently estimated to be in the order of $10 billion per year. Demographic trends, *i.e.*, the gradually increasing age of the US population, suggest that these costs may increase 2-3 fold by the year 2020 if a safe and

effective treatment is not found.

The major focus of current therapies for osteoporosis is fracture prevention, not fracture repair. This is an important consideration, as it is known that significant morbidity and mortality are associated with prolonged bed rest in the elderly, especially those who have suffered hip fracture. New methods are clearly needed for stimulating fracture repair, thus restoring mobility in these patients before the complications arise.

Osteogenesis imperfecta (OI) refers to a group of inherited connective tissue diseases characterized by bone and soft connective tissue fragility (Byers and Steiner, 1992; Prockop, 1990). Males and females are affected equally, and the overall incidence is currently estimated to be 1 in 5,000-14,000 live births. Hearing loss, dentinogenesis imperfecta, respiratory insufficiency, severe scoliosis and emphysema are just some of the conditions that are associated with one or more types of OI. While accurate estimates of the health care costs are not available, the morbidity and mortality associated with OI certainly result from the extreme propensity to fracture (OI types I-IV) and the deformation of abnormal bone following fracture repair (OI types II-IV) (Bonadio and Goldstein, 1993). The most relevant issue with OI treatment is to develop new methods by which to improve fracture repair and thus to improve the quality of life of these patients.

The techniques of bone reconstruction, such as is used to reconstruct defects occurring as a result of trauma, cancer surgery or errors in development, would also be improved by new methods to promote bone repair. Reconstructive methods currently employed, such as using autologous bone grafts, or bone grafts with attached soft

tissue and blood vessels, are associated with significant drawbacks of both cost and difficulty. For example, harvesting a useful amount of autologous bone is not easily achieved, and even autologous grafts often become infected or suffer from resorption.

The process of bone repair and regeneration resembles the process of wound healing in other tissues. A typical sequence of events includes; hemorrhage; clot formation; dissolution of the clot with concurrent removal of damaged tissues; ingrowth of granulation tissue; formation of cartilage; capillary ingrowth and cartilage turnover; rapid bone formation (callus tissue); and, finally, remodeling of the callus into cortical and trabecular bone. Therefore, bone repair is a complex process that involves many cell types and regulatory molecules. The diverse cell populations involved in fracture repair include stem cells, macrophages, fibroblasts, vascular cells, osteoblasts, chondroblasts, and osteoclasts.

Regulatory factors involved in bone repair are known to include systemic hormones, cytokines, growth factors, and other molecules that regulate growth and differentiation. Various osteoinductive agents have been purified and shown to be polypeptide growth-factor-like molecules. These stimulatory factors are referred to as bone morphogenetic or morphogenic proteins (BMPs), and have also been termed osteogenic bone inductive proteins or osteogenic proteins (OPs). Several BMP (or OP) genes have now been cloned, and the common designations are BMP-1 through BMP-8. New BMPs are in the process of discovery. Although the BMP terminology is widely used, it may prove to be the case that there is an OP

counterpart term for every individual BMP (Alper, 1994).

BMPs 2-8 are generally thought to be osteogenic, although BMP-1 is a more generalized morphogen (Shimell *et al.*, 1991). BMP-3 is also called osteogenin (Luyten *et al.*, 1989) and BMP-7 is also called OP-1 (Ozkaynak *et al.*, 1990). BMPs are related to, or part of, the transforming growth factor-β (TGF-β) superfamily, and both TGF-β1 and TGF-β2 also regulate osteoblast function (Seitz *et al.*, 1992). Several BMP (or OP) nucleotide sequences and polypeptides have been described in U.S. Patents, *e.g.*, 4,795,804; 4,877,864; 4,968,590; 5,108,753; including, specifically, BMP-1 disclosed in U.S. Patent 5,108,922; BMP-2A (currently referred to as BMP-2) in U.S. Patents 5,166,058 and 5,013,649; BMP-2B (currently referred to as BMP-4) disclosed in U.S. Patent 5,013,649; BMP-3 in 5,116,738; BMP-5 in 5,106,748; BMP-6 in 5,187,076; BMP-7 in 5,108,753 and 5,141,905; and OP-1, COP-5 and COP-7 in 5,011,691.

Other growth factors or hormones that have been reported to have the capacity to stimulate new bone formation include acidic fibroblast growth factor (Jingushi *et al.*, 1990); estrogen (Boden *et al.*, 1989); macrophage colony stimulating factor (Horowitz *et al.*, 1989); and calcium regulatory agents such as parathyroid hormone (PTH) (Raisz and Kream, 1983).

Several groups have investigated the possibility of using bone stimulating proteins and polypeptides, particularly recombinant BMPs, to influence bone repair *in vivo*. For example, recombinant BMP-2 has been employed to repair surgically created defects in the mandible of adult dogs (Toriumi *et al.*, 1991), and high doses of this molecule have been shown to functionally repair segmental defects in rat femurs (Yasko *et al.*, 1992). Chen and colleagues showed that a single

application of 25-100 mg of recombinant TGF-β1 adjacent to cartilage induced endochondral bone formation in the rabbit ear full thickness skin wounds (Chen *et al.*, 1991). It has also been reported that an application of TGF-β1 in a 3% methylcellulose gel was able to repair surgically induced large skull defects that otherwise heal by fibrous connective tissue and never form bone (Beck *et al.*, 1991).

However, there are many drawbacks associated with these type of treatment protocols, not least the expensive and time-consuming purification of the recombinant proteins from their host cells. Also, polypeptides, once administered to an animal are more unstable than is generally desired for a therapeutic agent, and they are susceptible to proteolytic attack. Furthermore, the administration of recombinant proteins can initiate various inhibitive or otherwise harmful immune responses. It is clear, therefore, that a new method capable of promoting bone repair and regeneration *in vivo* would represent a significant scientific and medical advance with immediate benefits to a large number of patients. A method readily adaptable for use with a variety of matrices and bone-stimulatory genes would be particularly advantageous.

SUMMARY OF THE INVENTION

The present invention overcomes one or more of these and other drawbacks inherent in the prior art by providing novel methods, compositions and devices for use in transferring nucleic acids into bone cells and tissues, and for promoting bone repair and regeneration. Certain embodiments of the invention rest, generally, with the inventors' surprising finding that nucleic acids can be effectively transferred to bone progenitor cells

in vivo and that, in certain embodiments, the transfer of an osteotropic gene stimulates bone repair in an animal.

The invention, in general terms, thus concerns methods, compositions and devices for transferring a nucleic acid segment into bone progenitor cells or tissues. The methods of the invention generally comprise contacting bone progenitor cells with a composition comprising a nucleic acid segment in a manner effective to transfer the nucleic acid segment into the cells. The cells may be cultured cells or recombinant cells maintained *in vitro*, when all that is required is to add the nucleic acid composition to the cells, *e.g.*, by adding it to the culture media.

Alternatively, the progenitor cells may be located within a bone progenitor tissue site of an animal, when the nucleic acid composition would be applied to the site in order to effect, or promote, nucleic acid transfer into bone progenitor cells *in vivo*. In transferring nucleic acids into bone cells within an animal, a preferred method involves first adding the genetic material to a bone-compatible matrix and then using the resultant matrix to contact an appropriate tissue site within the animal. The "resultant" matrix may, in certain embodiments, be referred to as a matrix impregnated with genetic material, or it may take the form of a matrix-nucleic acid mixture, or even conjugate.

An extremely wide variety of genetic material can be transferred to bone progenitor cells or tissues using the compositions and methods of the invention. For example, the nucleic acid segment may be DNA (double or single-stranded) or RNA (*e.g.*, mRNA, tRNA, rRNA); it may also be a "coding segment", *i.e.*, one that encodes a protein or polypeptide, or it may be an antisense nucleic acid molecule, such as antisense RNA that may function to

disrupt gene expression. The nucleic acid segments may thus be genomic sequences, including exons or introns alone or exons and introns, or coding cDNA regions, or in fact any construct that one desires to transfer to a bone progenitor cell or tissue. Suitable nucleic acid segments may also be in virtually any form, such as naked DNA or RNA, including linear nucleic acid molecules and plasmids; functional inserts within the genomes of various recombinant viruses, including viruses with DNA genomes and retroviruses; and any form of nucleic acid segment, plasmid or virus associated with a liposome or a gold particle, the latter of which may be employed in connection with the gene gun technology.

The invention may be employed to promote expression of a desired gene in bone cells or tissues and to impart a particular desired phenotype to the cells. This expression could be increased expression of a gene that is normally expressed (*i.e.*, "over-expression"), or it could be used to express a gene that is not normally associated with bone progenitor cells in their natural environment. Alternatively, the invention may be used to suppress the expression of a gene that is naturally expressed in such cells and tissues, and again, to change or alter the phenotype. Gene suppression may be a way of expressing a gene that encodes a protein that exerts a down-regulatory function, or it may utilize antisense technology.

1. Bone Progenitor Cells and Tissues

In certain embodiments, this invention provides advantageous methods for using genes to stimulate bone progenitor cells. As used herein, the term "bone progenitor cells" refers to any or all of those cells that have the capacity to ultimately form, or contribute to the formation of, new bone tissue. This includes

various cells in different stages of differentiation, such as, for example, stem cells, macrophages, fibroblasts, vascular cells, osteoblasts, chondroblasts, osteoclasts, and the like. Bone progenitor cells also include cells that have been isolated and manipulated *in vitro*, *e.g.*, subjected to stimulation with agents such as cytokines or growth factors or even genetically engineered cells. The particular type or types of bone progenitor cells that are stimulated using the methods and compositions of the invention are not important, so long as the cells are stimulated in such a way that they are activated and, in the context of *in vivo* embodiments, ultimately give rise to new bone tissue.

The term "bone progenitor cell" is also used to particularly refer to those cells that are located within, are in contact with, or migrate towards (*i.e.*, "home to"), bone progenitor tissue and which cells directly or indirectly stimulate the formation of mature bone. As such, the progenitor cells may be cells that ultimately differentiate into mature bone cells themselves, *i.e.*, cells that "directly" form new bone tissue. Cells that, upon stimulation, attract further progenitor cells or promote nearby cells to differentiate into bone-forming cells (*e.g.*, into osteoblasts, osteocytes and/or osteoclasts) are also considered to be progenitor cells in the context of this disclosure - as their stimulation "indirectly" leads to bone repair or regeneration. Cells affecting bone formation indirectly may do so by the elaboration of various growth factors or cytokines, or by their physical interaction with other cell types. Although of scientific interest, the direct or indirect mechanisms by which progenitor cells

stimulate bone or wound repair is not a consideration in practicing this invention.

Bone progenitor cells and bone progenitor tissues may be cells and tissues that, in their natural environment, arrive at an area of active bone growth, repair or regeneration (also referred to as a wound repair site). In terms of bone progenitor cells, these may also be cells that are attracted or recruited to such an area. These may be cells that are present within an artificially-created osteotomy site in an animal model, such as those disclosed herein. Bone progenitor cells may also be isolated from animal or human tissues and maintained in an *in vitro* environment. Suitable areas of the body from which to obtain bone progenitor cells are areas such as the bone tissue and fluid surrounding a fracture or other skeletal defect (whether or not this is an artificially created site), or indeed, from the bone marrow. Isolated cells may be stimulated using the methods and compositions disclosed herein and, if desired, be returned to an appropriate site in an animal where bone repair is to be stimulated. In such cases, the nucleic-acid containing cells would themselves be a form of therapeutic agent. Such *ex vivo* protocols are well known to those of skill in the art.

In important embodiments of the invention, the bone progenitor cells and tissues will be those cells and tissues that arrive at the area of bone fracture or damage that one desires to treat. Accordingly, in treatment embodiments, there is no difficulty associated with the identification of suitable target progenitor cells to which the present therapeutic compositions should be applied. All that is required in such cases is to obtain an appropriate stimulatory composition, as disclosed herein, and contact the site of the bone fracture or defect with the composition. The nature of

this biological environment is such that the appropriate cells will become activated in the absence of any further targeting or cellular identification by the practitioner.

Certain methods of the invention involve, generally, contacting bone progenitor cells with a composition comprising one or more osteotropic genes (with or without additional genes, proteins or other biomolecules) so as to promote expression of said gene in said cells. As outlined above, the cells may be contacted *in vitro* or *in vivo*. This is achieved, in the most direct manner, by simply obtaining a functional osteotropic gene construct and applying the construct to the cells. The present inventors surprisingly found that there are no particular molecular biological modifications that need to be performed in order to promote effective expression of the gene in progenitor cells. Contacting the cells with DNA, *e.g.*, a linear DNA molecule, or DNA in the form of a plasmid or other recombinant vector, that contains the gene of interest under the control of a promoter, along with the appropriate termination signals, is sufficient to result in uptake and expression of the DNA, with no further steps necessary.

In preferred embodiments, the process of contacting the progenitor cells with the osteotropic gene composition is conducted *in vivo*. Again, a direct consequence of this process is that the cells take up and express the gene and that they, without additional steps, function to stimulate bone tissue growth, repair or regeneration.

An assay of an osteoinductive gene may be conducted using the bone induction bioassay of Sampath and Reddi (1981; incorporated herein by reference). This is a rat bone formation assay that is routinely used to evaluate the osteogenic activity of bone inductive factors.

However, for analyzing the effects of osteotropic genes on bone growth, one is generally directed to use the novel osteotomy model disclosed herein.

2. Osteotropic Genes

As used herein, the terms "osteotropic and osteogenic gene" are used to refer to a gene or DNA coding region that encodes a protein, polypeptide or peptide that is capable of promoting, or assisting in the promotion of, bone formation, or one that increases the rate of primary bone growth or healing (or even a gene that increases the rate of skeletal connective tissue growth or healing). The terms promoting, inducing and stimulating are used interchangeably throughout this text to refer to direct or indirect processes that ultimately result in the formation of new bone tissue or in an increased rate of bone repair. Thus, an osteotropic gene is a gene that, when expressed, causes the phenotype of a cell to change so that the cell either differentiates, stimulates other cells to differentiate, attracts bone-forming cells, or otherwise functions in a manner that ultimately gives rise to new bone tissue.

In using the new osteotomy model of the invention, an osteotropic gene is characterized as a gene that is capable of stimulating proper bone growth in the osteotomy gap to any degree higher than that observed in control studies, *e.g.*, parallel studies employing an irrelevant marker gene such as β-galactosidase. This stimulation of "proper bone growth" includes both the type of tissue growth and the rate of bone formation. In using the model with a 5 mm osteotomy gap, an osteotropic gene is generally characterized as a gene that is capable of promoting or inducing new bone formation, rather than abnormal bone fracture repair, *i.e.*, fibrous non-union. In using the 2 mm osteotomy gap, one may characterize

osteotropic genes as genes that increase the rate of primary bone healing as compared to controls, and more preferably, genes capable of stimulating repair of the osteotomy defect in a time period of less than nine weeks.

In general terms, an osteotropic gene may also be characterized as a gene capable of stimulating the growth or regeneration of skeletal connective tissues such as, *e.g.*, tendon, cartilage, and ligament. Thus, in certain embodiments, the methods and compositions of the invention may be employed to stimulate the growth or repair of both bone tissue itself and also of skeletal connective tissues.

A variety of osteotropic genes are now known, all of which are suitable for use in connection with the present invention. Osteotropic genes and the proteins that they encode include, for example, systemic hormones, such as parathyroid hormone (PTH) and estrogen; many different growth factors and cytokines; chemotactic or adhesive peptides or polypeptides; molecules such as activin (U.S. Patent 5,208,219, incorporated herein by reference); specific bone morphogenetic proteins (BMPs); and even growth factor receptor genes.

Examples of suitable osteotropic growth factors include those of the transforming growth factor (TGF) gene family, including TGFs 1-3, and particularly TGF-β1, TGF-β2 and TGF-β3, (U.S. Patents 4,886,747 and 4,742,003, incorporated herein by reference), with TGF-α (U.S. Patent 5,168,051, incorporated herein by reference) also being of possible use; and also fibroblast growth factors (FGF), previously referred to as acidic and basic FGF and now referred to as FGF1-9; granulocyte/macrophage colony stimulating factor (GMCSF); epidermal growth factor (EGF); platelet derived growth factor (PDGF); insulin-

like growth factors (IGF), including IGF-I and IGF-II; and leukemia inhibitory factor (LIF), also known as HILDA and DIA. Any of the above or other related genes, or DNA segments encoding the active portions of such proteins, may be used in the novel methods and compositions of the invention.

Certain preferred osteotropic genes and DNA segments are those of the TGF superfamily, such as TGF-β1, TGF-β2, TGF-β3 and members of the BMP family of genes. For example, several BMP genes have been cloned that are ideal candidates for use in the nucleic acid transfer or delivery protocols of the invention. Suitable BMP genes are those designated BMP-2 through BMP-12. BMP-1 is not considered to be particularly useful at this stage.

There is considerable variation in the terminology currently employed in the literature in referring to these genes and polypeptides. It will be understood by those of skill in the art that all BMP genes that encode an active osteogenic protein are considered for use in this invention, regardless of the differing terminology that may be employed. For example, BMP-3 is also called osteogenin and BMP-7 is also called OP-1 (osteogenic protein-1). It is likely that the family of factors termed OP(s) is as large as that termed BMP(s), and that these terms, in fact, describe the same set of molecules (Alper, 1994).

The DNA sequences for several BMP (or OP) genes have been described both in scientific articles and in U.S. Patents such as 4,877,864; 4,968,590; 5,108,753. Specifically, BMP-1 sequences are disclosed in U.S. Patent 5,108,922; BMP-2A (currently referred to as BMP-2) in U.S. Patents 5,166,058 and 5,013,649; BMP-2B (currently referred to as BMP-4) disclosed in U.S. Patent 5,013,649; BMP-3 in 5,116,738; BMP-5 in 5,106,748; BMP-6

in 5,187,076; and BMP-7 in 5,108,753 and 5,141,905; all incorporated herein by reference). The article by Wozney *et al.*, (1988; incorporated herein by reference) is considered to be particularly useful for describing BMP molecular clones and their activities. DNA sequences encoding the osteogenic proteins designated OP-1, COP-5 and COP-7 are also disclosed in U.S. Patent 5,011,691.

All of the above issued U.S. Patents are incorporated herein by reference and are intended to be used in order to supplement the present teachings regarding the preparation of BMP and OP genes and DNA segments that express osteotropic polypeptides. As disclosed in the above patents, and known to those of skill in the art, the original source of a recombinant gene or DNA segment to be used in a therapeutic regimen need not be of the same species as the animal to be treated. In this regard, it is contemplated that any recombinant PTH, TGF or BMP gene may be employed to promote bone repair or regeneration in a human subject or an animal, *e.g.*, a horse. Particularly preferred genes are those from human, murine and bovine sources, in that such genes and DNA segments are readily available, with the human or murine forms of the gene being most preferred for use in human treatment regimens. Recombinant proteins and polypeptides encoded by isolated DNA segments and genes are often referred to with the prefix "r" for recombinant and "rh" for recombinant human. As such, DNA segments encoding rBMPs, such as rhBMP-2 or rhBMP-4, are contemplated to be particularly useful in connection with this invention.

The definition of a "BMP gene", as used herein, is a gene that hybridizes, under relatively stringent hybridization conditions (see, *e.g.*, Maniatis *et al.*, 1982), to DNA sequences presently known to include BMP gene sequences.

To prepare an osteotropic gene segment or cDNA one may follow the teachings disclosed herein and also the teachings of any of patents or scientific documents specifically referenced herein. Various nucleotide sequences encoding active BMPs are disclosed in U.S. Patents 5,166,058, 5,013,649, 5,116,738, 5,106,748, 5,187,076, 5,108,753 and 5,011,691, each incorporated herein by reference. By way of example only, U.S. Patent 5,166,058, teaches that hBMP-2 is encoded by a nucleotide sequence from nucleotide #356 to nucleotide #1543 of the sequence shown in Table II of the patent. One may thus obtain a hBMP-2 DNA segment using molecular biological techniques, such as polymerase chain reaction (PCR™) or screening a cDNA or genomic library, using primers or probes with sequences based on the above nucleotide sequence. The practice of such techniques is a routine matter for those of skill in the art, as taught in various scientific articles, such as Sambrook *et al.*, (1989), incorporated herein by reference. Certain documents further particularly describe suitable mammalian expression vectors, *e.g.*, U.S. Patent 5,168,050, incorporated herein by reference.

Osteotropic genes and DNA segments that are particularly preferred for use in certain aspects of the present compositions and methods are the TGF, PTH and BMP genes. TGF genes are described in U.S. Patents 5,168,051; 4,886,747 and 4,742,003, each incorporated herein by reference. TGFα may not be as widely applicable as TGFβ, but is proposed for use particularly in applications involving skeletal soft tissues. The PTH gene, or a DNA segment encoding the active fragment thereof, such as a DNA segment encoding a polypeptide that includes the amino acids 1-34 (hPTH1-34; Hendy *et al.*, 1981; incorporated herein by reference) is another

preferred gene; as are the BMP genes termed BMP-4 and BMP-2, such as the gene or cDNA encoding the murine BMP-4 disclosed herein.

It is also contemplated that one may clone further genes or cDNAs that encode an osteotropic protein or polypeptide. The techniques for cloning DNA molecules, *i.e.*, obtaining a specific coding sequence from a DNA library that is distinct from other portions of DNA, are well known in the art. This can be achieved by, for example, screening an appropriate DNA library, as disclosed in Example XV herein, which relates to the cloning of a wound healing gene. The screening procedure may be based on the hybridization of oligonucleotide probes, designed from a consideration of portions of the amino acid sequence of known DNA sequences encoding related osteogenic proteins. The operation of such screening protocols are well known to those of skill in the art and are described in detail in the scientific literature, for example, in Sambrook *et al.*, (1989), incorporated herein by reference.

Osteotropic genes with sequences that vary from those described in the literature are also encompassed by the invention, so long as the altered or modified gene still encodes a protein that functions to stimulate bone progenitor cells in any direct or indirect manner. These sequences include those caused by point mutations, those due to the degeneracies of the genetic code or naturally occurring allelic variants, and further modifications that have been introduced by genetic engineering, *i.e.*, by the hand of man.

Techniques for introducing changes in nucleotide sequences that are designed to alter the functional properties of the encoded proteins or polypeptides are well known in the art, *e.g.*, U.S. Patent 4,518,584,

incorporated herein by reference, which techniques are also described in further detail herein. Such modifications include the deletion, insertion or substitution of bases, and thus, changes in the amino acid sequence. Changes may be made to increase the osteogenic activity of a protein, to increase its biological stability or half-life, to change its glycosylation pattern, and the like. All such modifications to the nucleotide sequences are encompassed by this invention.

It will, of course, be understood that one or more than one osteotropic gene may be used in the methods and compositions of the invention. The nucleic acid delivery methods may thus entail the administration of one, two, three, or more, osteotropic genes. The maximum number of genes that may be applied is limited only by practical considerations, such as the effort involved in simultaneously preparing a large number of gene constructs or even the possibility of eliciting a significant adverse cytotoxic effect. The particular combination of genes may be two or more distinct BMP genes; or it may be such that a growth factor gene is combined with a hormone gene, *e.g.*, a BMP gene and a PTH gene; a hormone or growth factor gene may even be combined with a gene encoding a cell surface receptor capable of interacting with the polypeptide product of the first gene.

In using multiple genes, they may be combined on a single genetic construct under control of one or more promoters, or they may be prepared as separate constructs of the same of different types. Thus, an almost endless combination of different genes and genetic constructs may be employed. Certain gene combinations may be designed to, or their use may otherwise result in, achieving synergistic effects on cell stimulation and bone growth,

any and all such combinations are intended to fall within the scope of the present invention. Indeed, many synergistic effects have been described in the scientific literature, so that one of ordinary skill in the art would readily be able to identify likely synergistic gene combinations, or even gene-protein combinations.

It will also be understood that, if desired, the nucleic segment or gene could be administered in combination with further agents, such as, *e.g.*, proteins or polypeptides or various pharmaceutically active agents. So long as genetic material forms part of the composition, there is virtually no limit to other components which may also be included, given that the additional agents do not cause a significant adverse effect upon contact with the target cells or tissues. The nucleic acids may thus be delivered along with various other agents, for example, in certain embodiments one may wish to administer an angiogenic factor, and/or an inhibitor of bone resorption, as disclosed in U.S. Patents 5,270,300 and 5,118,667, respectively, each incorporated herein by reference.

3. Gene Constructs and DNA Segments

As used herein, the terms "gene" and "DNA segment" are both used to refer to a DNA molecule that has been isolated free of total genomic DNA of a particular species. Therefore, a gene or DNA segment encoding an osteotropic gene refers to a DNA segment that contains sequences encoding an osteotropic protein, but is isolated away from, or purified free from, total genomic DNA of the species from which the DNA is obtained. Included within the term "DNA segment", are DNA segments and smaller fragments of such segments, and also recombinant vectors, including, for example, plasmids, cosmids, phage, retroviruses, adenoviruses, and the like.

The term "gene" is used for simplicity to refer to a functional protein or peptide encoding unit. As will be understood by those in the art, this functional term includes both genomic sequences and cDNA sequences. "Isolated substantially away from other coding sequences" means that the gene of interest, in this case, an osteotropic gene, forms the significant part of the coding region of the DNA segment, and that the DNA segment does not contain large portions of naturally-occurring coding DNA, such as large chromosomal fragments or other functional genes or cDNA coding regions. Of course, this refers to the DNA segment as originally isolated, and does not exclude genes or coding regions, such as sequences encoding leader peptides or targeting sequences, later added to the segment by the hand of man.

This invention provides novel ways in which to utilize various known osteotropic DNA segments and recombinant vectors. As described above, many such vectors are readily available, one particular detailed example of a suitable vector for expression in mammalian cells is that described in U.S. Patent 5,168,050, incorporated herein by reference. However, there is no requirement that a highly purified vector be used, so long as the coding segment employed encodes a osteotropic protein and does not include any coding or regulatory sequences that would have a significant adverse effect on bone progenitor cells. Therefore, it will also be understood that useful nucleic acid sequences may include additional residues, such as additional non-coding sequences flanking either of the 5' or 3' portions of the coding region or may include various internal sequences, *i.e.*, introns, which are known to occur within genes.

After identifying an appropriate osteotropic gene or DNA molecule, it may be inserted into any one of the many vectors currently known in the art, so that it will

direct the expression and production of the osteotropic protein when incorporated into a bone progenitor cell. In a recombinant expression vector, the coding portion of the DNA segment is positioned under the control of a promoter. The promoter may be in the form of the promoter which is naturally associated with an osteotropic gene, as may be obtained by isolating the 5' non-coding sequences located upstream of the coding segment or exon, for example, using recombinant cloning and/or PCR™ technology, in connection with the compositions disclosed herein.

In other embodiments, it is contemplated that certain advantages will be gained by positioning the coding DNA segment under the control of a recombinant, or heterologous, promoter. As used herein, a recombinant or heterologous promoter is intended to refer to a promoter that is not normally associated with an osteotropic gene in its natural environment. Such promoters may include those normally associated with other osteotropic genes, and/or promoters isolated from any other bacterial, viral, eukaryotic, or mammalian cell. Naturally, it will be important to employ a promoter that effectively directs the expression of the DNA segment in bone progenitor cells.

The use of recombinant promoters to achieve protein expression is generally known to those of skill in the art of molecular biology, for example, see Sambrook *et al.*, (1989). The promoters employed may be constitutive, or inducible, and can be used under the appropriate conditions to direct high level or regulated expression of the introduced DNA segment. The currently preferred promoters are those such as CMV, RSV LTR, the SV40 promoter alone, and the SV40 promoter in combination with various enhancer elements.

Osteotropic genes and DNA segments may also be in the form of a DNA insert which is located within the genome of a recombinant virus, such as, for example a recombinant adenovirus, adeno-associated virus (AAV) or retrovirus. In such embodiments, to place the gene in contact with a bone progenitor cell, one would prepare the recombinant viral particles, the genome of which includes the osteotropic gene insert, and simply contact the progenitor cells or tissues with the virus, whereby the virus infects the cells and transfers the genetic material.

In certain preferred embodiments, one would impregnate a matrix or implant material with virus by soaking the material in recombinant virus stock solution, *e.g.*, for 1-2 hours, and then contact the bone progenitor cells or tissues with the resultant, impregnated matrix. Cells then penetrate, or grow into, the matrix, thereby contacting the virus and allowing viral infection which leads to the cells taking up the desired gene or cDNA and expressing the encoded protein.

In other preferred embodiments, one would form a matrix-nucleic acid admixture, whether using naked DNA, a plasmid or a viral vector, and contact the bone progenitor cells or tissues with the resultant admixed matrix. The matrix may then deliver the nucleic acid into the cells following disassociation at the cell surface, or in the immediate cellular environment. Equally, the matrix admixture itself, especially a particle- or fiber-DNA admixture, may be taken up by cells to provide subsequent intracellular release of the genetic material. The matrix may then be extruded from the cell, catabolized by the cell, or even stored within the cell. The molecular mechanism by which a bone-compatible matrix achieves transfer of DNA to a cell is immaterial to the practice of the present invention.

4. **Bone-Compatible Matrices**

In certain preferred embodiments, the methods of the invention involved preparing a composition in which the osteotropic gene, genes, DNA segments, or cells already incorporating such genes or segments, are associated with, impregnated within, or even conjugated to, a bone-compatible matrix, to form a "matrix-gene composition" and the matrix-gene composition is then placed in contact with the bone progenitor cells or tissue. The matrix may become impregnated with a gene DNA segment simply by soaking the matrix in a solution containing the DNA, such as a plasmid solution, for a brief period of time of anywhere from about 5 minutes or so, up to and including about two weeks.

Matrix-gene compositions are all those in which genetic material is adsorbed, absorbed, impregnated, conjugated to, or otherwise generally maintained in contact with the matrix. "Maintained in contact with the matrix" means that an effective amount of the nucleic acid composition should remain functionally associated with the matrix until its transfer to the bone progenitor cell or its release in the bone tissue site.

The type of matrix that may be used in the compositions, devices and methods of the invention is virtually limitless, so long as it is a "bone-compatible matrix". This means that the matrix has all the features commonly associated with being "biocompatible", in that it is in a form that does not produce a significant adverse, allergic or other untoward reaction when administered to an animal, and that it is also suitable for placing in contact with bone tissue. A "significant" adverse effect is one that exceeds the normally accepted side-effects associated with any given therapy.

"Bone-compatible", as used herein, means that the matrix (and gene) does not produce a significant adverse or untoward reaction when placed in contact with bone. In certain embodiments, when electing to use a particular bone compatible matrix, one may, optionally, take various other factors into consideration, for example, the capacity of the matrix to provide a structure for the developing bone, its capacity to be resorbed into the body after the bone has been repaired, and such like. However, these properties are not required to practice the invention and are merely exemplary of the factors that may be considered.

In other embodiments, one may also consider the likelihood that the matrix will be transported into the cell, e.g., by active or passive membrane transport. Where such transport and subsequent nucleic acid release is contemplated, other properties of the matrix and gene may be assessed in optimizing the matrix-gene formulation. For example, adenovirus vectors may provide for advantageous DNA release in such embodiments. Matrices that are readily metabolized in the cytoplasm would also likely be preferred in such embodiments. Matrices that are later released from the cell, and preferably, also removed from the surrounding tissue area, would be another preferred form of matrix for use in such embodiments.

The choice of matrix material will differ according to the particular circumstances and the site of the bone that is to be treated. Matrices such as those described in U.S. Patent 5,270,300 (incorporated herein by reference) may be employed. Physical and chemical characteristics, such as, *e.g.*, biocompatibility, biodegradability, strength, rigidity, interface properties, and even cosmetic appearance, may be considered in choosing a matrix, as is well known to

those of skill in the art. Appropriate matrices will deliver the gene composition and, in certain circumstances, may be incorporated into a cell, or may provide a surface for new bone growth, *i.e.*, they may act as an *in situ* scaffolding through which progenitor cells may migrate.

A particularly important aspect of the present invention is its use in connection with orthopaedic implants and interfaces and artificial joints, including implants themselves and functional parts of an implant, such as, *e.g.*, surgical screws, pins, and the like. In preferred embodiments, it is contemplated that the metal surface or surfaces of an implant or a portion thereof, such as a titanium surface, will be coated with a material that has an affinity for nucleic acids, most preferably, with hydroxyl apatite, and then the coated-metal will be further coated with the gene or nucleic acid that one wishes to transfer. The available chemical groups of the absorptive material, such as hydroxyl apatite, may be readily manipulated to control its affinity for nucleic acids, as is known to those of skill in the art.

In certain embodiments, non-biodegradable matrices may be employed, such as sintered hydroxylapatite, aluminates, other bioceramic materials and metal materials, particularly titanium. A suitable ceramic delivery system is that described in U.S. Patent 4,596,574, incorporated herein by reference. Polymeric matrices may also be employed, including acrylic ester polymers, lactic acid polymers, and polylactic polyglycolic acid (PLGA) block copolymers, have been disclosed (U.S. Patent 4,526,909, U.S. Patent 4,563,489, Simons *et al.*, 1992, and Langer and Folkman, 1976, respectively, each incorporated herein by reference).

In certain embodiments, it is contemplated that a biodegradable matrix will likely be most useful. A biodegradable matrix is generally defined as one that is capable of being resorbed into the body. Potential biodegradable matrices for use in connection with the compositions, devices and methods of this invention include, for example, biodegradable and chemically defined calcium sulfate, tricalciumphosphate, hydroxylapatite, PLGA block copolymers, polyanhydrides, matrices of purified proteins, and semi-purified extracellular matrix compositions.

One preferred group of matrices are collagenous matrices, including those obtained from tendon or dermal collagen, *e.g.*, type I collagen, which is generally prepared from dermis; those obtained from cartilage, such as type II collagen; and various other types of collagen. Collagens may be obtained from a variety of commercial sources, *e.g.*, Sigma that supplies type II collagen obtained from bovine trachea; and Collagen Corporation. Collagen matrices may also be prepared as described in U.S. Patents 4,394,370 and 4,975,527, each incorporated herein by reference.

The various collagenous materials may also be in the form of mineralized collagen. One preferred mineralized collagenous material is that termed UltraFiber™, obtainable from Norian Corp. (Mountain View, CA). U.S. Patent 5,231,169, incorporated herein by reference, describes the preparation of mineralized collagen through the formation of calcium phosphate mineral under mild agitation *in situ* in the presence of dispersed collagen fibrils. Such a formulation may be employed in the

context of delivering a nucleic acid segment to a bone tissue site.

Certain other preferred collagenous materials are those based upon type II collagen. Type II collagen preparations have been discovered to have the surprising and advantageous property of, absent any osteotropic gene, being capable of stimulating bone progenitor cells. Prior to the present invention, it was thought that type II collagen only had a structural role in the cartilage extracellular matrix and the present finding that type II collagen is actually an osteoconductive/osteoinductive material is unexpected. The present invention thus contemplates the use of a variety of type II collagen preparations as gene transfer matrices or bone cell stimulants, either with or without DNA segments, including native type II collagen, as prepared from cartilage, and recombinant type II collagen.

PLGA block copolymers may also be employed as gene transfer matrices. Such polymers have been shown to readily incorporate DNA, are commercially available, non-toxic, and hydrolyze at defined rates, (*i.e.* they facilitate the sustained release of pharmaceutical agents). PLGA block copolymers have two particular advantageous properties in that, first, they exhibit reversible thermal gelation, and second, may be combined with other agents that allow for radiographic visualization.

5. Nucleic Acid Transfer Embodiments

Once a suitable matrix-gene composition has been prepared or obtained, all that is required to deliver the osteotropic gene to bone progenitor cells within an animal is to place the matrix-gene composition in contact

with the site in the body in which one wishes to promote bone growth. This may be achieved by physically positioning the matrix-gene composition in contact with the body site, or by injecting a syringeable form of the matrix-gene composition into the appropriate area.

The matrix-gene composition may be applied to a simple bone fracture site that one wishes to repair, an area of weak bone, such as in a patient with osteoporosis, or a bone cavity site that one wishes to fill with new bone tissue. Bone cavities may arise as a result of an inherited disorder, birth defect, or may result following dental or periodontal surgery or after the removal of an osteosarcoma.

The use of PLGA and like compounds as matrices allows the matrix-DNA composition to be syringeable, which is achieved by, generally, admixing the matrix-gene composition with a pluronic agent. The resultant matrix-gene-pluronic may be stored within a thermal-jacket syringe, maintained at a temperature of about 4°C, immediately prior to administration to the body. In this temperature and environment, the composition will be a liquid. Following insertion into the body, the composition will equilibrate towards body temperature, and in so-doing will form a gelatinous matrix.

The above phenomenon is termed "reversible thermal gelation", and this allows for a controlled rate of gelation to be achieved. The manner of using pluronic agents in this context will be known to those of skill in the art in light of the present disclosure. Matrix-gene-pluronic compositions may also be admixed, or generally associated with, an imaging agent so that the present gene transfer technology may be used in imaging modalities. In these cases, the attending physician or veterinarian will be able to monitor the delivery and

positioning of the matrix-gene composition. Many safe and effective imaging agents, such as the radiographic compound calcium phosphate, are available that may be used in conjunction with fluoroscopy, or even with tomography, to image the body or tissue site while the composition is being delivered.

Where an image of the tissue site is to be provided, one will desire to use a detectable imaging agent, such as a radiographic agent, or even a paramagnetic or radioactive agent. Many radiographic diagnostic agents are known in the art to be useful for imaging purposes, including *e.g.*, calcium phosphate.

In the case of paramagnetic ions, examples include chromium (III), manganese (II), iron (III), iron (II), cobalt (II), nickel (II), copper (II), neodymium (III), samarium (III), ytterbium (III), gadolinium (III), vanadium (II), terbium (III), dysprosium (III), holmium (III) and erbium (III), with gadolinium being generally preferred. Ions useful in other contexts, such as X-ray imaging, include but are not limited to, lanthanum (III), gold (III), lead (II), and especially bismuth (III).

Although not generally preferred, radioactive isotopes are not excluded and may be used for imaging purposes if desired. Suitable ions include iodine^{131}, iodine^{123}, technicium^{99m}, indium^{111}, rhenium^{188}, rhenium^{186}, gallium^{67}, copper^{67}, yttrium^{90}, iodine^{125} and astatine^{211}.

The amount of gene construct that is applied to the matrix and the amount of matrix-gene material that is applied to the bone tissue will be determined by the attending physician or veterinarian considering various biological and medical factors. For example, one would wish to consider the particular osteotropic gene and matrix, the amount of bone weight desired to be formed,

the site of bone damage, the condition of the damaged bone, the patient's or animal's age, sex, and diet, the severity of any infection, the time of administration and any further clinical factors that may affect bone growth, such as serum levels of various factors and hormones. The suitable dosage regimen will therefore be readily determinable by one of skill in the art in light of the present disclosure, bearing in mind the individual circumstances.

In treating humans and animals, progress may be monitored by periodic assessment of bone growth and/or repair, *e.g.*, using X-rays. The therapeutic methods and compositions of the invention are contemplated for use in both medical and veterinary applications, due to the lack of species specificity in bone inductive factors. In particular, it is contemplated that domestic, farm and zoological animals, as well as thoroughbred horses, would be treatable using the nucleic acid transfer protocols disclosed herein.

The present methods and compositions may also have prophylactic uses in closed and open fracture reduction and also in the improved fixation of artificial joints. The invention is applicable to stimulating bone repair in congenital, trauma-induced, or oncologic resection-induced craniofacial defects, and also is useful in the treatment of periodontal disease and other tooth repair processes and even in cosmetic plastic surgery. The matrix-gene compositions and devices of this invention may also be used in wound healing and related tissue repair, including, but not limited to healing of burns, incisions and ulcers.

The present invention also encompasses DNA-based compositions for use in cellular transfer to treat bone defects and disorders. The compositions of the invention

generally comprise an osteotropic gene in association with a bone-compatible matrix, such as type II collagen, wherein the composition is capable of stimulating bone growth, repair or regeneration upon administration to, or implantation within, a bone progenitor tissue site of an animal. The osteotropic gene or genes may be any of those described above, with TGF-α (for soft skeletal tissues), TGF-β1, TGF-β2, TGF-β3, PTH, BMP-2 and BMP-4 genes being generally preferred. Likewise, irrespective of the choice of gene, the bone-compatible matrix may be any of those described above, with biodegradable matrices such as collagen and, more particularly, type II collagen, being preferred.

In still further embodiments, the present invention concerns osteotropic devices, which devices may be generally considered as molded or designed matrix-gene compositions. The devices of the invention naturally comprise a bone-compatible matrix in which an osteotropic gene is associated with the matrix. The combination of genes and matrix components is such that the device is capable of stimulating bone growth or healing when implanted in an animal. The devices may be of virtually any size or shape, so that their dimensions are adapted to fit a bone fracture or bone cavity site in the animal that is to be treated, allowing the fracture join and/or bone regrowth to be more uniform. Other particularly contemplated devices are those that are designed to act as an artificial joint. Titanium devices and hydroxylapatite-coated titanium devices will be preferred in certain embodiments. Parts of devices in combination with an osteotropic nucleic acid segment, such as a DNA-coated screw for an artificial joint, and the like, also fall within the scope of the invention.

Therapeutic kits comprising, in suitable container means, a bone compatible matrix, such as type II collagen

or a PLGA block polymer, and an osteotropic gene form another aspect of the invention. Such kits will generally contain a pharmaceutically acceptable formulation of the matrix and a pharmaceutically acceptable formulation of an osteotropic gene, such as PTH, BMP, TGF-β, FGF, GMCSF, EGF, PDGF, IGF or a LIF gene. Currently preferred genes include PTH, TGF-β1, TGF-β2, TGF-β3, and BMP-4 genes.

The kits may comprise a single container means that contains both the biocompatible matrix and the osteotropic gene. The container means may, if desired, contain a pharmaceutically acceptable sterile syringeable matrix, having associated with it, the osteotropic gene composition and, optionally, a detectable label or imaging agent. The syringeable matrix-DNA formulation may be in the form of a gelatinous composition, *e.g.*, a type II collagen-DNA composition, or may even be in a more fluid form that nonetheless forms a gel-like composition upon administration to the body. In these cases, the container means may itself be a syringe, pipette, or other such like apparatus, from which the matrix-DNA material may be applied to a bone tissue site or wound area. However, the single container means may contain a dry, or lyophilized, mixture of a matrix and osteotropic gene composition, which may or may not require pre-wetting before use.

Alternatively, the kits of the invention may comprise distinct container means for each component. In such cases, one container would contain the osteotropic gene, either as a sterile DNA solution or in a lyophilized form, and the other container would include the matrix, which may or may not itself be pre-wetted with a sterile solution, or be in a gelatinous, liquid or other syringeable form.

The kits may also comprise a second or third container means for containing a sterile, pharmaceutically acceptable buffer, diluent or solvent. Such a solution may be required to formulate either the DNA component, the matrix component, both components separately, or a pre-mixed combination of the components, into a more suitable form for application to the body, *e.g.*, a more gelatinous form. It should be noted, however, that all components of a kit could be supplied in a dry form (lyophilized), which would allow for "wetting" upon contact with body fluids. Thus, the presence of any type of pharmaceutically acceptable buffer or solvent is not a requirement for the kits of the invention. The kits may also comprise a second or third container means for containing a pharmaceutically acceptable detectable imaging agent or composition.

The container means will generally be a container such as a vial, test tube, flask, bottle, syringe or other container means, into which the components of the kit may placed. The matrix and gene components may also be aliquoted into smaller containers, should this be desired. The kits of the present invention may also include a means for containing the individual containers in close confinement for commercial sale, such as, *e.g.*, injection or blow-molded plastic containers into which the desired vials or syringes are retained.

Irrespective of the number of containers, the kits of the invention may also comprise, or be packaged with, an instrument for assisting with the placement of the ultimate matrix-gene composition within the body of an animal. Such an instrument may be a syringe, pipette, forceps, or any such medically approved delivery vehicle.

6. Type II Collagen as an Osteoconductive/inductive Material

The present invention also provides methods for stimulating bone progenitor cells, as may be applied, in certain circumstances, to promote new bone formation, or to stimulate wound-healing. As such, the bone progenitor cells that are the targets of the invention may also be termed "wound healing bone progenitor cells". Although the function of wound healing itself may not always be required to practice all aspects of the invention, and although a mechanistic understanding is not necessary to practice the invention, it is generally thought that the wound healing process does operate during execution of the invention.

To stimulate a bone progenitor cell in accordance with these aspects of the invention, generally one would contact a bone progenitor cell with a composition comprising a biologically effective amount of type II collagen. Although preparations of crushed bone and mineralized collagen have been shown to be osteoconductive, this property has not previously been ascribed to type II collagen. The present inventors have found that type II collagen alone is surprisingly effective at promoting new bone formation, it being able to bridge a 5 mm osteotomy gap in only eight weeks in all animals tested (FIG. 5A, FIG. 5B, FIG. 6A, FIG. 6B, FIG. 6C, FIG. 6D, FIG. 7A, FIG. 7B, FIG. 8A, FIG. 8B, and FIG. 8C).

The forms of type II collagen that may be employed in this invention are virtually limitless. For example, type II collagen may be purified from hyaline cartilage of bovine trachea, or as isolated from diarthrodial joints or growth plates. Purified type II collagen is commercially available and may be purchased from, *e.g.*, Sigma Chemical Company, St. Louis, MO. Any form of

recombinant type II collagen may also be employed, as may be obtained from a type II collagen-expressing recombinant host cell, including bacterial, yeast, mammalian, and insect cells. One particular example of a recombinant type II collagen expression system is a yeast cell that includes an expression vector that encodes type II collagen, as disclosed herein in Example VI.

The type II collagen used in the invention may, if desired, be supplemented with additional minerals, such as calcium, *e.g.*, in the form of calcium phosphate. Both native and recombinant type II collagen may be supplemented by admixing, adsorbing, or otherwise associating with, additional minerals in this manner. Such type II collagen preparations are clearly distinguishable from the types of "mineralized collagen" previously described, *e.g.*, in U.S. Patent 5,231,169 that describes the preparation of mineralized total collagen fibrils.

An object of this aspect of the invention is to provide a source of osteoconductive matrix material, that may be reproducibly prepared in a straightforward and cost-effective manner, and that may be employed, with or without an osteotropic gene segment, to stimulate bone progenitor cells. Recombinant type II collagen was surprisingly found to satisfy these criteria. Although clearly not required for effective results, the combination of native or recombinant type II collagen with mineral supplements, such as calcium, is encompassed by this invention.

A biologically effective amount of type II collagen is an amount of type II collagen that functions to stimulate a bone progenitor cell, as described herein. By way of example, one measure of a biologically effective amount is an amount effective to stimulate bone

progenitor cells to the extent that new bone formation is evident. In this regard, the inventors have shown that 10 mg of lyophilized collagen functions effectively to close a 5 mm osteotomy gap in three weeks. This information may be used by those of skill in the art to optimize the amount of type II collagen needed for any given situation.

Depending on the individual case, the artisan would, in light of this disclosure, readily be able to calculate an appropriate amount, or dose, of type II collagen for stimulating bone cells and promoting bone growth. In terms of small animals or human subjects, suitable effective amounts of collagen include between about 1 mg and about 500 mg, and preferably, between about 1 mg and about 100 mg, of lyophilized type II collagen per bone tissue site. Of course, it is likely that there will be variations due to, *e.g.*, individual responses, particular tissue conditions, and the speed with which bone formation is required. While 10 mg were demonstrated to be useful in the illustrative example, the inventors contemplate that 1, 5, 10, 15, 20, 30, 40, 50, 75, 100, 125, 150, 200, 300 mg, and the like, may be usefully employed for human patients and small animals. Of course, any values within these contemplated ranges may be useful in any particular case.

Naturally, one of the main variables to be accounted for is the amount of new bone that needs to be generated in a particular area or bone cavity. This can be largely a function of the size of the animal to be treated, e.g., a cat or a horse. Therefore, there is currently no upper limit on the amount of type II collagen, or indeed on the amount of any matrix-gene composition, that can be employed in the methods of the invention, given careful supervision by the practitioner.

In contacting or applying type II collagen, with or without a DNA segment, to bone progenitor cells located within a bone progenitor tissue site of an animal, bone tissue growth will be stimulated. Thus, bone cavity sites and bone fractures may be filled and repaired.

The use of type II collagen in combination with a nucleic acid segment that encodes a polypeptide or protein that stimulates bone progenitor cells when expressed in said cells is preferred, as described above. Nucleic acid segments that comprise an isolated PTH gene, BMP gene, growth factor gene, growth factor receptor gene, cytokine gene or a chemotactic factor gene are preferred, with PTH, TGF-β and BMP genes being most preferred. The genes function subsequent to their transfer into, and expression in, bone progenitor cells of the treated animal, thus promoting bone growth.

Although type II collagen alone is effective, its combined use with an osteotropic gene segment may prove to give synergistic and particularly advantageous effects. Type II collagen, whether native or recombinant, may thus also be formulated into a therapeutic kit with an osteotropic gene segment, in accordance with those kits described herein above. This includes the use of single or multiple container means, and combination with any medically approved delivery vehicle, including, but not limited to, syringes, pipettes, forceps, additional diluents, and the like.

BRIEF DESCRIPTION OF THE DRAWINGS

The drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings

in combination with the detailed description of specific embodiments presented herein.

FIG. 1. A model of DNA therapy for bone repair.

FIG. 2A. A schematic model of the cellular and molecular basis of the direct DNA transfer mechanism into osteogenic cells *in vivo*. Shown is the method of creating osteotomy and placing gene-activated matrix *in situ*.

FIG. 2B. A schematic model of the cellular and molecular basis of the direct DNA transfer mechanism into osteogenic cells *in vivo*. Shown is the method of fracturing repair cells where blood vessels grow into the gene-activated matrix (FIG. 2A).

FIG. 2C. A schematic model of the cellular and molecular basis of the direct DNA transfer mechanism into osteogenic cells *in vivo*. Shown are fractured cells taking up DNA as an episomal element, *i.e.* direct gene transfer *in vivo*.

FIG. 2D. A schematic model of the cellular and molecular basis of the direct DNA transfer mechanism into osteogenic cells *in vivo*. Shown are fractured repair synthesizing and secreting recombinant proteins encoded by the episomal DNA.

FIG. 2E. A schematic model of the cellular and molecular basis of the direct DNA transfer mechanism into osteogenic cells *in vivo*. Shown is the resulting new bone formation.

FIG. 3A. Achilles' tendon gene transfer is shown as a time course overview at 3 weeks post-surgery.

FIG. 3B. Achilles' tendon gene transfer is shown as a time course overview at 9 weeks post-surgery.

FIG. 3C. Achilles' tendon gene transfer is shown as a time course overview at 12 weeks post-surgery.

FIG. 3D. Achilles' tendon gene transfer is shown as a time course immunohistochemistry study. Shown is the microscopy of tendon tissue that received SIS implant impregnated with expression plasmid DNA. Note the positive cytoplasmic staining of fibroblastic cells 9 weeks post-surgery.

FIG. 3E. Achilles' tendon gene transfer is shown as a time course immunohistochemistry study. Shown is the microscopy of tendon tissue that received SIS implant alone, without DNA. Note the relative absence of cytoplasmic staining.

FIG. 4. Monitoring of cruciate ligament gene transfer using a substrate utilization assay. Three weeks following the implantation of SIS soaked in a solution of the pSV40β-gal expression plasmid, tendon tissue was harvested, briefly fixed in 0.5% glutaraldehyde, and then incubated with X-gal according to published methods. Tissues were then embedded in paraffin and sections were cut and stained with H and E. Note the positive (arrows) staining in the cytoplasm of granulation tissues fibroblasts.

FIG. 5A. Direct DNA transfer into regenerating bone: β-gal activity. The figure compares β-galactosidase activity in homogenates of osteotomy gap tissue from two Sprague-Dawley rats. In animal #1, the UltraFiber™ implant material was soaked in a solution of pSV40β-gal DNA, Promega) encoding bacterial β-galactosidase. In animal #2, the implant material was

soaked in a pure solution of pGL2-Promoter Vector DNA (Promega) encoding insect luciferase. Enzyme activity was determined using substrate assay kits (β-galactosidase and Luciferase Assay Systems, Promega). Note that significant β-galactosidase activity was found only in the homogenate prepared from animal #1.

FIG. 5B. Direct DNA transfer into regenerating bone: luciferase activity. The figure compares luciferase activity in aliquots of the homogenates described in FIG. 5A. Luciferase activity was determined using the commercial reagents and protocols (Promega) described in FIG. 5A. Note that significant luciferase activity is found only in the homogenate prepared from animal #2.

FIG. 6A. Osteotomy gene transfer monitored by PTH studies. In this study an expression plasmid coding for a functional 34 amino acid peptide fragment of human parathyroid hormone (PTH1-34) was transferred and expressed *in vivo* using the GAM technology. The progress of new bone formation in the gap was monitored radiographically for three weeks and the animals were sacrificed. Shown is a radiograph of the osteotomy gap of the control animal that received an antisense hPTH1-34 GAM construct. There was no evidence of radiodense tissue in the gap.

FIG. 6B. Osteotomy gene transfer (FIG. 6A) monitored by PTH studies. Shown is a histological section of osteotomy repair tissue from the same control animal. The section is characterized by the presence of granulation tissue fibroblasts and capillaries.

FIG. 6C. Osteotomy gene transfer (FIG. 6A) monitored by PTH studies. Shown is a radiograph of the osteotomy gap that received the sense PTH1-34 GAM

construct. Note the presence of radiodense tissue in the gap (arrow).

FIG. 6D. Osteotomy gene transfer (FIG. 6A) monitored by PTH studies. Shown is a histological section of osteotomy repair tissue from the same control animal. The section is characterized by the presence of trabecular bone plates that extend into the gap from the surgical margin.

FIG. 7A. Osteotomy gene transfer BMP-4 studies. Shown is immunohistochemical evidence of BMP-4 transgene expression by granulation tissue fibroblasts near the center of an osteotomy gap three weeks after surgery. Note the positive (arrows) staining of spindled cells. The BMP-4 transgene included an epitope tag (HA epitope, Pharmacia) that facilitated the identification of transgenic BMP-4 molecules. Tissue staining was performed using commercially available polyclonal anti-HA antibodies and standard procedures. Immunostaining was localized only to gap tissues. Control sections included serial sections stained with pre-immune rabbit serum and tissue sections from 13 control osteotomy gaps. In both instances all controls were negative for peroxidase staining of granulation tissue fibroblasts.

FIG. 7B. Osteotomy gene transfer BMP-4 studies. Shown is the histology of newly formed bone as early as three weeks following gene transfer (FIG. 7A).

FIG. 8A. Radiographic evidence of bridging new bone formation (arrows) as a consequence of BMP-4 gene transfer and expression at six weeks' post surgery. 9 and 16 weeks post-op, are presented in FIG. 8B and FIG. 8C, respectively, to demonstrate the orderly growth of new bone *in situ* over time. This animal, which has been maintained for 23 weeks, has been ambulating normally

without an external fixator for the past 7 weeks. Similar results have been obtained in a second long term animal (of two) that is now 17 weeks post-op.

FIG. 8B. Radiographic evidence of bridging new bone formation (arrows) as a consequence of BMP-4 gene transfer and expression at nine weeks' post surgery (see FIG. 8A).

FIG. 8C. Radiographic evidence of bridging new bone formation (arrows) as a consequence of BMP-4 gene transfer and expression at sixteen weeks' post surgery (see FIG. 8A).

FIG. 9A. The animal shown here is representative of the control group that received an osteotomy plus a collagen sponge without DNA of any type. The animal was maintained for 9 weeks following surgery and then sacrificed. Progress of new bone formation in the gap was monitored radiographically and histologically. Shown is a radiograph of the osteotomy gap at 9 weeks. Note the absence of radiodense tissue in the gap.

FIG. 9B. Shown is a histological section of osteotomy gap tissue from the control animal used in FIG 9A. The section is characterized by the presence of granulation tissue fibroblasts and capillaries.

FIG. 10. PLJ-HPTH1-34 expression construct. A cDNA fragment coding for a prepro-hPTH1-34 peptide was generated by PCR™ (Hendy *et al.*, 1981) and then ligated into a *Bam*HI cloning site in the PLJ retroviral expression vector (Wilson *et al.*, 1992). Several independent clones with the insert in the coding orientation were isolated and characterized.

FIG. 11. Southern analysis of retroviral

integration in the YZ-15 clone. 10 mg of YZ-15 genomic DNA were digested with *Kpn*I (for which there is a unique site in the vector LTR) and analyzed by Southern blotting. A cDNA fragment that coded for prepro-hPTH1-35 was used as a probe. The positive control for the Southern hybridization conditions was a *Kpn*I digest of genomic DNA from Rat-1 cells infected and selected with the recombinant, replication-defective retrovirus PLJ-hPTH1-84 (Wilson *et al.*, 1992). *Kpn*I digests of DNA were also prepared from two negative controls: native Rat-1 cells and Rat-1 cells infected and selected with BAG ("BAG cells", (Wilson *et al.*, 1992), a replication-defective recombinant retrovirus that encodes β-galactosidase, which is an irrelevant marker gene in these studies. Lane assignments were as follows: 1, PLJ-hPTH1-84 cells; 2 BAG cells; 3, YZ-15; 4, native Rat-1 cells. DNA sizes (kb) are shown at the left of the figure. As expected, a fragment of the predicted size (*e.g.*, 4.3 kb) is seen only in lane 1 (the positive control) and in lane 3 (YZ-15 DNA).

FIG. 12. Northern blot analysis of a transduced Rat-1 clone. Poly-A($^+$)RNA was prepared from the YZ-15 clone and analyzed by Northern blotting as described (Chen *et al.*, 1993). FIG. 12 contains two panels on a single sheet. Poly-A($^+$) RNA prepared from PLJ-hPTH1-84 cells, BAG cells, and native Rat-1 cells were used as positive and negative controls. Four probes were applied to a single blot following sequential stripping: hPTH1-34, β-gal, *Neo*, and β-actin. Lane assignments were as follows: 1, PLJ-hPTH1-84 cells; 2, BAG cells; 3, YZ-15 cells; 4, native Rat-1 cells. As expected, the hPTH1-34 transcript is seen only in lane 1 (positive control) and in lane 3-4; a *Neo* transcript is seen in lanes 1-3; a β-gal transcript is seen only in lane 2; and β-actin transcripts are seen in lanes 1-4.

FIG. 13. Northern analysis of poly-A(+) RNA demonstrating expression of the PTH/PTHrP receptor in osteotomy repair tissue.

FIG. 14. Overlapping murine cDNA clones representing the LTBP-like (LTBP-3) sequence. A partial representation of restriction sites is shown. N, *Nco*I; P, *Pvu*II; R, *Rsa*II; B, *Bam*HI; H, *Hin*dIII. The numbering system at the bottom assumes that the "A" of the initiator Met codon is nt #1.

FIG. 15A. A schematic showing the structure of the murine fibrillin-1 gene product. Structural domains are shown below the diagram. Symbols designating various structural elements are defined in the legend to FIG. 15B.

FIG. 15B. A schematic showing the structure of the murine LTBP-like (LTBP-3) molecule. Domains #1-5 are denoted below the diagram. Symbols designate the following structural elements: EGF-CB repeats: open rectangles; TGF-bp repeats: open ovals; Fib motif: open circle; TGF-bp-like repeat: patterned oval; cysteine-rich sequences: patterned rectangles; proline/glycine-rich region: thick curved line, domain #2; proline-rich region, thick curved line, domain #3. Note that symbols designating the signal peptide have been deleted for simplicity. Additionally, the schematic assumes that EGF-like and EGF-CB repeats may extend for several amino acids beyond the C_6 position.

FIG. 15C. A schematic showing the structure of human LTBP-1. Domains #1-5 are denoted below the diagram. The symbols designating the structural elements are defined in the legend to FIG. 15B.

FIG. 16. Overview of expression of the new LTBP-

like (LTBP-3) gene during murine development as determined by tissue *in situ* hybridization. FIG. 16 consists of autoradiograms made by direct exposure of tissue sections to film after hybridization with radiolabeled probes. Day 8.5-9.0 sections contained embryos surrounded by intact membranes, uterine tissues, and the placental disk, cut in random planes. Day 13.5 and 16.5 sections contain isolated whole embryos sectioned in the sagittal plane near or about the mid-line. Identical conditions were maintained throughout autoradiography and photography, thereby allowing a comparison of the overall strength of hybridization in all tissue sections. The transcript is expressed in connective tissue, mesenchyme, liver, heart and CNS.

FIG. 17A. Selected microscopic views of mouse LTBP-3 gene expression in day 8.5-9.0 p.c. mouse developing tissues. All photographs in FIG. 17A- FIG. 17D were taken from the same slides used to prepare whole mount sections (after dipping slides in radiographic emulsion). Shown is the neural tube, brightfield image. 1 cm = 20 mm.

FIG. 17B. Selected microscopic views of mouse LTBP-3 gene expression in day 8.5-9.0 p.c. mouse developing tissues. Shown is the neural tube, darkfield image. Note expression by neuroepithelial cells and by surrounding mesenchyme. 1 cm = 20 mm.

FIG. 17C. Selected microscopic views of mouse LTBP-3 gene expression in day 8.5-9.0 p.c. mouse developing tissues. Shown is the heart, brightfield image. The figure demonstrates expression by myocardial and endocardial (arrowheads) cells. 1 cm = 20 mm.

FIG. 17D. Selected microscopic views of mouse LTBP-3 gene expression in day 8.5-9.0 p.c. mouse

developing tissues. Shown is the heart, darkfield image. The figure demonstrates expression by myocardial and endocardial (arrowheads) cells. Darkfield photomicrographs were taken after exposure of tissues to photographic emulsion for 2 weeks. In this image and the one shown in FIG. 17B, red blood cell and other plasma membranes give a faint white signal that contributes to the background of the experiment. 1 cm = 20 mm.

FIG. 18A. Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. All photographs in FIG. 18A - FIG. 18P were taken from the same slides used to prepare whole mount sections (after dipping slides in radiographic emulsion). Shown is the cartilage model of developing long bone from lower extremity, brightfield image. Expression by chondrocytes and by perichondrial cells is seen in FIG. 18B. 1 cm = 20 mm.

FIG. 18B. Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the cartilage model of developing long bone from lower extremity, darkfield image. Note expression by chondrocytes and by perichondrial cells. In all darkfield views of FIG. 18, red blood cell and other plasma membranes give a faint white signal that contributes to the background of the experiment. Note the absence of spurious hybridization signal in areas of the slide that lack cellular elements. 1 cm = 20 mm.

FIG. 18C. Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the lung, brightfield image. 1 cm = 20 mm.

FIG. 18D. Microscopy of mouse LTBP-3 gene

expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the heart, brightfield image. 1 cm = 20 mm.

FIG. 18E. Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the lung, darkfield image. Note expression by epithelial cells of developing airway and by the surrounding parenchymal cells. 1 cm = 20 mm.

FIG. 18F. Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the heart, darkfield image. Note continuing expression by myocardial cells. 1 cm = 20 mm.

FIG. 18G. Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the pancreas, brightfield image. 1 cm = 20 mm.

FIG. 18H. Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the intestine, brightfield image. 1 cm = 20 mm.

FIG. 18I. Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the pancreas, darkfield image. Note expression by acinar epithelial cells. 1 cm = 20 mm.

FIG. 18J. Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is intestine, darkfield image. Note the expression in epithelial and subepithelial cells. 1 cm = 20 mm.

FIG. 18K. Microscopy of mouse LTBP-3 gene

expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is kidney, brightfield image. 1 cm = 20 mm.

FIG. 18L. Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is skin, brightfield image. 1 cm = 20 mm.

FIG. 18M. Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is kidney, darkfield image. Note expression by blastemal cells beneath the kidney capsule, epithelial cells of developing nephrons and tubules, and the interstitial mesenchyme. 1 cm = 20 mm.

FIG. 18N. Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the skin, darkfield image. Note the expression by epidermal, adnexal and dermal cells of developing skin. 1 cm = 20 mm.

FIG. 18O. Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the retina, brightfield image. 1 cm = 20 mm.

FIG. 18P. Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the retina, darkfield image. Note expression by retinal epithelial cells and by adjacent connective tissue cells. 1 cm = 20 mm.

FIG. 19. Time-dependent expression of the LTBP-3 gene by MC3T3-E1 cells. mRNA preparation and Northern blotting were preformed as described in Example XIV. Equal aliquots of total RNA as determined by UV

spectroscopy were loaded in each lane of the Northern gel. As demonstrated by UV spectroscopy were loaded in each lane of the Northern gel. As demonstrated by methylene blue staining (Sambrook *et al.*, 1989), equal amounts of RNA were transferred to the nylon membrane. The results demonstrate a clear, strong peak in LTBP-3 gene expression by 14 days in culture. Weaker signals denoting LTBP-3 gene expression also can be observed after 5 days and 28 days in culture.

FIG. 20. Antisera #274 specifically binds LTBP-3 epitopes. Transfection of 293T cells with a full length mouse LTBP-3 expression plasmid followed by radiolabeling, preparation of medium sample, immunoprecipitation, and 4-18% gradient SDS-PAGE were performed as described in Example XIV. The figure presents a SDS-PAGE autoradiogram of medium samples following a 2 day exposure to film. Lane assignments are as follows: Lane 1, radiolabeled 293T medium (prior to transfection) immunoprecipitated with preimmune serum; Land 2, radiolabeled 293T medium (prior to transfection) immunoprecipitated with antibody #274; Lane 3, radiolabeled 393T medium (following transfection and preincubation with 10 μg of LTBP-3 synthetic peptide cocktail) immunoprecipitated with antibody #274; and Lane 4, radiolabeled 293T medium (following transfection) immunoprecipitated with antibody #274. As indicated by the bar, the full length LTBP-3 molecule migrated at 180-190 kDa.

FIG. 21. Co-immunoprecipitation of LTBP-3 and TGR-β1 produced by MC3T3-E1 cells. Aliquots (~10^6 incorporated CPM) of radiolabeled media produced by MC3T3-E1 cells after 7 days in culture were immunoprecipitated as described in Example XIV. Bars indicate the position of cold molecular weight standards used to estimate molecular weight (Rainbow mix,

Amersham). Immunoprecipitates were separated using 4%-18% gradient SDS-PAGE and reducing conditions. The figure shows a negative control lane 1 consisting of MC3T3-E1 medium immunoprecipitated with anti-LTBP-3 antibody #274. Western blotting was performed using the lower portion of the gradient gel and a commercially available antibody to TGF-β1 (Santa Cruz Biotechnology, Inc.). Antibody staining was detected using commercially available reagents and protocols (ECL Western Blotting Reagent, Amersham). MC3T3-E1 medium was immunoprecipitated with anti-LTBP-2 antibody #274.

FIG. 22A. Radiographic analysis of the type II collagen osteotomy gap three weeks after surgery.

FIG. 22B. Radiographic analysis of the type I collagen osteotomy gap three weeks after surgery.

FIG. 22C. Histologic analysis of the type II collagen osteotomy shown in FIG. 22A.

FIG. 23A. Adenovirus-mediated gene transfer into bone repair/regeneration cells *in vivo*. Positive (arrows) β-gal cytoplasmic staining is observed in the fracture repair cells.

FIG. 23B. Adenovirus-mediated gene transfer into bone repair/regeneration cells *in vivo*. Serial section negative control strained with the vehicle of the β-gal antibody plus a cocktail of non-specific rabbit IgG antibodies.

FIG. 23C. Adenovirus-mediated gene transfer into bone repair/regeneration cells *in vivo*. Osteotomy site was filled with a fibrous collagen implant material soaked in a solution of the replication-defective recombinant adenovirus AdRSVβ-gal ($\sim 10^{11}$ plaque forming

units/ml). Note the positive (arrow) β-gal nuclear staining of chondrocytes within the osteotomy site, as demonstrated by immunohistochemistry using a specific anti-β-gal antibody.

FIG. 24. The murine BMP-4 amino acid sequence, SEQ ID NO:1. The HA epitope is shown in bold at the extreme carboxy terminus of the sequence.

FIG. 25. DNA sequence of the murine LTBP-3 gene (SEQ ID NO:2).

FIG. 26. Amino acid sequence of the murine LTBP-3 gene product (SEQ ID NO:3).

FIG. 27. DNA sequence of the murine LTBP-2 gene (SEQ ID NO:17).

FIG. 28. Amino acid sequence of the murine LTBP-2 gene product (SEQ ID NO:18).

DESCRIPTION OF THE PREFERRED EMBODIMENT

1. Applications of Bone Repair Technology to Human Treatment

The following is a brief discussion of four human conditions to exemplify the variety of diseases and disorders that would benefit from the development of new technology to improve bone repair and healing processes. In addition to the following, several other conditions, such as, for example, vitamin D deficiency; wound healing in general; soft skeletal tissue repair; and cartilage and tendon repair and regeneration, may also benefit from technology concerning the stimulation of bone progenitor cells.

The first example is the otherwise healthy individual who suffers a fracture. Often, clinical bone fracture is treated by casting to alleviate pain and allow natural repair mechanisms to repair the wound. While there has been progress in the treatment of fracture in recent times, even without considering the various complications that may arise in treating fractured bones, any new procedures to increase bone healing in normal circumstances would represent a great advance.

A second example which may benefit from new treatment methods is osteogenesis imperfecta (OI). OI encompasses various inherited connective tissue diseases that involve bone and soft connective tissue fragility in humans (Byers and Steiner, 1992; Prockop, 1990). About one child per 5,000-20,000 born is affected with OI and the disease is associated with significant morbidity throughout life. A certain number of deaths also occur, resulting in part from the high propensity for bone fracture and the deformation of abnormal bone after fracture repair (OI types II-IV; Bonadio and Goldstein, 1993). The relevant issue here is quality of life; clearly, the lives of affected individuals would be improved by the development of new therapies designed to stimulate and strengthen the fracture repair process.

OI type I is a mild disorder characterized by bone fracture without deformity, blue sclerae, normal or near normal stature, and autosomal dominant inheritance (Bonadio and Goldstein, 1993). Osteopenia is associated with an increased rate of lone bone fracture upon ambulation (the fracture frequency decreases dramatically at puberty and during young adult life, but increases once again in late middle age). Hearing loss, which often begins in the second or third decade, is a feature of this disease in about half the families and can

progress despite the general decline in fracture frequency. Dentinogenesis imperfecta is observed in a subset of individuals.

In contrast, OI types II-VI represent a spectrum of more severe disorders associated with a shortened life-span. OI type II, the perinatal lethal form, is characterized by short stature, a soft calvarium, blue sclerae, fragile skin, a small chest, floppy appearing lower extremities (due to external rotation and abduction of the femurs), fragile tendons and ligaments, bone fracture with severe deformity, and death in the perinatal period due to respiratory insufficiency. Radiographic signs of bone weakness include compression of the femurs, bowing of the tibiae, broad and beaded ribs, and calvarial thinning.

OI type III is characterized by short stature, a triangular facies, severe scoliosis, and bone fracture with moderate deformity. Scoliosis can lead to emphysema and a shortened life-span due to respiratory insufficiency. OI type IV is characterized by normal sclerae, bone fracture with mild to moderate deformity, tooth defects, and a natural history that essentially is intermediate between OI type II and OI type I.

More than 200 OI mutations have been characterized since 1989 (reviewed in Byers and Steiner, 1992; Prockop, 1990). The vast majority occur in the COL1A1 and COL1A2 genes of type I collagen. Most cases of OI type I appear to result from heterozygous mutations in the COL1A1 gene that decrease collagen production but do not alter primary structure, *i.e.*, heterozygous null mutations affecting COL1A1 expression. Most cases of OI types II-IV result from heterozygous mutations in the COL1A1 and COL1A2 genes that alter the structure of collagen.

A third important example is osteoporosis. The term osteoporosis refers to a heterogeneous group of disorders characterized by decreased bone mass and fractures. An estimated 20-25 million people are at increased risk for fracture because of site-specific bone loss. Risk factors for osteoporosis include increasing age, gender (more females), low bone mass, early menopause, race (Caucasians), low calcium intake, reduced physical activity, genetic factors, environmental factors (including cigarette smoking and abuse of alcohol or caffeine), and deficiencies in neuromuscular control that create a propensity to fall.

More than a million fractures in the USA each year can be attributed to osteoporosis, and in 1986 alone the treatment of osteoporosis cost an estimated 7-10 billion health care dollars. Demographic trends (*i.e.*, the gradually increasing age of the US population) suggest that these costs may increase 2-3 fold by the year 2020 if a safe and effective treatment is not found. Clearly, osteoporosis is a significant health care problem.

Clinically, osteoporosis is segregated into type I and type II. Type I osteoporosis occurs predominantly in middle aged women and is associated with estrogen loss at the menopause, while osteoporosis type II is associated with advancing age. Much of the morbidity and mortality associated with osteoporosis results from immobilization of elderly patients following fracture.

Current therapies for osteoporosis patients focus on fracture prevention, not fracture repair. This remains an important consideration because of the literature, which clearly states that significant morbidity and mortality are associated with prolonged bed rest in the elderly, particularly those who have suffered hip fractures. Complications of bed rest include blood clots

and pneumonia. These complications are recognized and measures are usually taken to avoid them, but these measures hardly represent the best approach to therapy. Thus, the osteoporotic patient population would benefit from new therapies designed to strengthen bone and speed up the fracture repair process, thereby getting these people on their feet before the complications arise.

A fourth example is related to bone reconstruction and, specifically, the ability to reconstruct defects in bone tissue that result from traumatic injury; cancer or cancer surgery; birth defect; a developmental error or heritable disorder; or aging. There is a significant orthopaedic need for more stable total joint implants, and cranial and facial bone are particular targets for this type of reconstructive need. The availability of new implant materials, *e.g.*, titanium, has permitted the repair of relatively large defects. Titanium implants provide excellent temporary stability across bony defects. However, experience has shown that a lack of viable bone bridging the defect can result in exposure of the appliance, infection, structural instability and, ultimately, failure to repair the defect.

Autologous bone grafts are another possible reconstructive modality, but they have several demonstrated disadvantages in that they must be harvested from a donor site such as iliac crest or rib, they usually provide insufficient bone to completely fill the defect, and the bone that does form is sometimes prone to infection and resorption. Partially purified xenogeneic preparations are not practical for clinical use because microgram quantities are purified from kilograms of bovine bone, making large scale commercial production both costly and impractical. Allografts and demineralized bone preparations are therefore often employed.

Microsurgical transfers of free bone grafts with attached soft tissue and blood vessels can close bony defects with an immediate source of blood supply to the graft. However, these techniques are time consuming, have been shown to produce a great deal of morbidity, and can only be used by specially trained individuals. Furthermore, the bone implant is often limited in quantity and is not readily contoured. In the mandible, for example, the majority of patients cannot wear dental appliances using presently accepted techniques (even after continuity is established), and thus gain little improvement in the ability to masticate. Toriumi *et al.*, have written that, "reconstructive surgeons should have at their disposal a bone substitute that would be reliable, biocompatible, easy to use, and long lasting and that would restore mandibular continuity with little associated morbidity."

In connection with bone reconstruction, specific problem areas for improvement are those concerned with treating large defects, such as created by trauma, birth defects, or particularly, following tumor resection. The success of orthopaedic implants, interfaces and artificial joints could conceivably be improved if the surface of the implant, or a functional part of an implant, were to be coated with a bone stimulatory agent. The surface of implants could be coated with one or more appropriate materials in order to promote a more effective interaction with the biological site surrounding the implant and, ideally, to promote tissue repair.

2. Bone Repair

Bone tissue is known to have the capacity for repair and regeneration and there is a certain understanding of the cellular and molecular basis of these processes. The

initiation of new bone formation involves the commitment, clonal expansion, and differentiation of progenitor cells. Once initiated, bone formation is promoted by a variety of polypeptide growth factors, Newly formed bone is then maintained by a series of local and systemic growth and differentiation factors.

The concept of specific bone growth-promoting agents is derived from the work of Huggins and Urist. Huggins *et al.*, 1936, demonstrated that autologous transplantation of canine incisor tooth to skeletal muscle resulted in local new bone formation (Huggins *et al.*, 1936). Urist and colleagues reported that demineralized lyophilized bone segments induced bone formation (Urist, 1965; Urist *et al.*, 1983), a process that involved macrophage chemotaxis; the recruitment of progenitor cells; the formation of granulation tissue, cartilage, and bone; bone remodeling; and marrow differentiation. The initiation of cartilage and bone formation in an extraskeletal site, a process referred to as osteoinduction, has permitted the unequivocal identification of initiators of bone morphogenesis (Urist, 1965; Urist *et al.*, 1983; Sampath *et al.*, 1984; Wang *et al.*, 1990; Cunningham *et al.*, 1992).

Significant progress has now been made in characterizing the biological agents elaborated by active bone tissue during growth and natural bone healing. Demineralized bone matrix is highly insoluble; Sampath and Reddi (1981) showed that only 3% of the proteins can be extracted using strong combinations of denaturants and detergents. They also showed that the unfractionated demineralized bone extract will initiate bone morphogenesis, a critical observation that led to the purification of "osteoinductive" molecules. Families of proteinaceous osteoinductive factors have now been purified and characterized. They have been variously

referred to in the literature as bone morphogenetic or morphogenic proteins (BMPs), osteogenic bone inductive proteins or osteogenic proteins (OPs).

3. Bone Repair and Bone Morphogenetic Proteins (BMPs)

Following their initial purification, several bone morphogenetic protein genes have now been cloned using molecular techniques (Wozney *et al.*, 1988; Rosen *et al.*, 1989; summarized in Alper, 1994). This work has established BMPs as members of the transforming growth factor-β (TGF-β) superfamily based on DNA sequence homologies. Other TGF molecules have also been shown to participate in new bone formation, and TGF-β is regarded as a complex multifunctional regulator of osteoblast function (Centrella *et al.*, 1988; Carrington *et al.*, 1988; Seitz *et al.*, 1992). Indeed, the family of transforming growth factors (TGF-β1, TGF-β2, and TGF-β3) has been proposed as potentially useful in the treatment of bone disease (U.S. Patent 5,125,978, incorporated herein by reference).

The cloning of distinct BMP genes has led to the designation of individual BMP genes and proteins as BMP-1 through BMP-8. BMPs 2-8 are generally thought to be osteogenic (BMP-1 may be a more generalized morphogen; Shimell *et al.*, 1991). BMP-3 is also called osteogenin (Luyten *et al.*, 1989) and BMP-7 is also called OP-1 (Ozkaynak *et al.*, 1990). TGFs and BMPs each act on cells via complex, tissue-specific interactions with families of cell surface receptors (Roberts and Sporn, 1989; Paralkar *et al.*, 1991).

Several BMP (or OP) nucleotide sequences and vectors, cultured host cells and polypeptides have been described in the patent literature. For example, U.S. Patents, 4,877,864, 4,968,590 and 5,108,753 all concern

osteogenic factors. More specifically, BMP-1 is disclosed in U.S. Patent 5,108,922; BMP-2 species, including MBP-2A and BMP-2B, are disclosed in U.S. Patents 5,166,058, 5,013,649, and 5,013,649; BMP-3 in 5,116,738; BMP-5 in 5,106,748; BMP-6 in 5,187,076; and BMP-7 in 5,108,753 and 5,141,905; all incorporated herein by reference. Various BMP clones and their activities are particularly described by Wozney *et al.*, (1988; incorporated herein by reference). DNA sequences encoding the osteogenic proteins designated OP-1, COP-5 and COP-7 are also disclosed in U.S. Patent 5,011,691. Although the BMP terminology is widely used, it may prove to be the case that there is an OP counterpart term for every individual BMP (Alper, 1994).

4. Bone Repair and Growth Factors and Cytokines

Transforming growth factors (TGFs) have a central role in regulating tissue healing by affecting cell proliferation, gene expression, and matrix protein synthesis (Roberts and Sporn, 1989). While not necessarily a direct effect, Bolander and colleagues have provided evidence that TGF-β1 and TGF-β2 can initiate both chondrogenesis and osteogenesis (Joyce *et al.*, 1990; Izumi *et al.*, 1992; Jingushi *et al.*, 1992). In these studies new cartilage and bone formation appeared to be dose dependent (*i.e.*, dependent on the local growth factor concentration). The data also suggested that TGF-β1 and TGF-β2 stimulated cell differentiation by a similar mechanism, even though they differed in terms of the ultimate amount of new cartilage and bone that was formed.

Other growth factors/hormones besides TGF and BMP may influence new bone formation following fracture. Bolander and colleagues injected recombinant acidic fibroblast growth factor into a rat fracture site

(Jingushi *et al.*, 1990). The major effect of multiple high doses (1.0 mg/50 ml) was a significant increase in cartilage tissue in the fracture gap, while lower doses had no effect. These investigators also used the reverse transcriptase-polymerase chain reaction (PCR™) technique to demonstrate expression of estrogen receptor transcripts in callus tissue (Boden *et al.*, 1989). These results suggested a role for estrogen in normal fracture repair.

Horowitz and colleagues have shown that activated osteoblasts will synthesize the cytokine, macrophage colony stimulating factor (Horowitz *et al.*, 1989). The osteotropic agents used in this study included lipopolysaccharide, PTH1-84, PTH1-34, vitamin D and all-trans retinoic acid. This observation has led to the suggestion that osteoblast activation following fracture may lead to the production of cytokines that regulate both hematopoiesis and new bone formation. Various other proteins and polypeptides that have been found to be expressed at high levels in osteogenic cells, such as, *e.g.*, the polypeptide designated Vgr-1 (Lyons *et al.*, 1989), also have potential for use in connection with the present invention.

5. Bone Repair and Calcium Regulating Hormones

Calcium regulating hormones such as parathyroid hormone (PTH) participate in new bone formation and bone remodeling (Raisz and Kream, 1983). PTH is an 84 amino acid calcium-regulating hormone whose principle function is to raise the Ca^{+2} concentration in plasma and extracellular fluid. Studies with the native hormone and with synthetic peptides have demonstrated that the amino-terminus of the molecule (aa 1-34) contains the structural requirements for biological activity (Tregear *et al.*, 1973; Hermann-Erlee *et al.*, 1976; Riond, 1993).

PTH functions by binding to a specific cell surface receptor that belongs to the G protein-coupled receptor superfamily (Silve *et al.*, 1982; Rizzoli *et al.*, 1983; Juppner *et al.*, 1991).

Using a retroviral approach, a human full-length PTH gene construct has been introduced into cultured rat fibroblasts to create recombinant PTH-secreting cells. These cells were then transplanted into syngeneic rat recipients that were observed to develop hypercalcemia mediated by the increased serum concentrations of PTH (Wilson *et al.*, 1992). The object of these studies was to create an animal model of primary hyperparathyroidism.

PTH has a dual effect on new bone formation, a somewhat confusing aspect of hormone function despite intensive investigation. PTH has been shown to be a potent direct inhibitor of type I collagen production by osteoblasts (Kream *et al.*, 1993). Intact PTH was also shown to stimulate bone resorption in organ culture over 30 years ago, and the hormone is known to increase the number and activity of osteoclasts. Recent studies by Gay and colleagues have demonstrated binding of [^{125}I]PTH(1-84) to osteoclasts in tissue sections and that osteoclasts bind intact PTH in a manner that is both saturable and time- and temperature dependent (Agarwala and Gay, 1992). While these properties are consistent with the presence of PTH/PTHrP receptors on the osteoclast cell surface, this hypothesis is still considered controversial. A more accepted view, perhaps, is that osteoclast activation occurs via an osteoblast signaling mechanism.

On the other hand, osteosclerosis may occur in human patients with primary hyperparathyroidism (Seyle, 1932). It is well known that individuals with hyperparathyroidism do not inexorably lose bone mass, but

eventually achieve a new bone remodeling steady state after an initial period of net bone loss. Chronic, low dose administration of the amino-terminal fragment of PTH (aa 1-34) also can induce new bone formation according to a time- and dose-dependent schedule (Seyle, 1932; Parsons and Reit, 1974).

Human PTH1-34 has recently been shown to: stimulate DNA synthesis in chick osteoblasts and chondrocytes in culture (van der Plas, 1985; Schluter *et al.*, 1989; Somjen *et al.*, 1990); increase bone cell number *in vivo* (Malluche *et al.*, 1986); enhance the *in vitro* growth of chick embryonic cartilage and bone (Kawashima, 1980; Burch and Lebovitz, 1983; Lewinson and Silbermann, 1986; Endo *et al.*, 1980; Klein-Nulend *et al.*, 1990); enhance surface bone formation (both cortical and trabecular bone) in normal and osteogenic animals and in humans with osteoporosis (Reeve *et al.*, 1976; Reeve *et al.*, 1980; Tam *et al.*, 1982; Hefti *et al.*, 1982; Podbesek *et al.*, 1983; Stevenson and Parsons, 1983; Slovik *et al.*, 1986; Gunness-Hey and Hock, 1984; Tada *et al.*, 1988; Spencer *et al.*, 1989; Hock and Fonseca, 1990; Liu and Kalu, 1990; Hock and Gera, 1992; Mitlak *et al.*, 1992; Ejersted *et al.*, 1993); and delay and reverse the catabolic effects of estrogen deprivation on bone mass (Hock *et al.*, 1988; Hori *et al.*, 1988; Gunness-Hey and Hock, 1989; Liu *et al.*, 1991). Evidence of synergistic interactions between hPTH-1-34 and other anabolic molecules has been presented, including insulin-like growth factor, BMP-2, growth hormone, vitamin D, and TGF-β (Slovik *et al.*, 1986; Spencer *et al.*, 1989; Mitlak *et al.*, 1992; Canalis *et al.*, 1989; Linkhart and Mohan, 1989; Seitz *et al.*, 1992; Vukicevic *et al.*, 1989).

Anecdotal observation has shown that serum PTH levels may be elevated following bone fracture (Meller *et al.*, 1984; Johnston *et al.*, 1985; Compston *et al.*, 1989;

Hardy *et al.*, 1993), but the significance of this observation is not understood. There are apparently no reports in the literature concerning attempts to localize either PTH or the PTH/PTHrP receptor *in situ* in human fracture sites or in experimental models. Furthermore, no attempt has been made to augment bone repair by the exogenous addition of PTH peptides. Although hPTH1-34 is known to function as an anabolic agent for bone, prior to the present invention, much remained to be learned about the role (if any) of PTH during bone regeneration and repair.

6. Protein Administration and Bone Repair

Several studies have been conducted in which preparations of protein growth factors, including BMPs, have been administered to animals in an effort to stimulate bone growth. The results of four such exemplary studies are described blow.

Toriumi *et al.*, studied the effect of recombinant BMP-2 on the repair of surgically created defects in the mandible of adult dogs (Toriumi *et al.*, 1991). Twenty-six adult hounds were segregated into three groups following the creation of a 3 cm full thickness mandibular defect: 12 animals received test implants composed of inactive dog bone matrix carrier and human BMP-2, 10 animals received control implants composed of carrier without BMP-2, and BMP-4 animals received no implant. The dogs were euthanized at 2.5-6 months, and the reconstructed segments were analyzed by radiography, histology, histomorphometry, and biomechanical testing. Animals that received test implants were euthanized after 2.5 months because of the presence of well mineralized bone bridging the defect. The new bone allowed these animals to chew a solid diet, and the average bending strength of reconstructed mandibles was 27% of normal

('normal' in this case represents the unoperated, contralateral hemimandible). In contrast, the implants in the other two groups were non-functional even after 6 months and showed minimal bone formation.

Yasko *et al.*, published a related study in which the effect of BMP-2 on the repair of segmental defects in the rat femur was examined (Yasko *et al.*, 1992). The study design included a group that received a dose of 1.4 mg of BMP-2, another group that received 11.0 mg of BMP-2, and a control group that received carrier matrix alone. Endochondral bone formation was observed in both groups of animals that received BMP-2. As demonstrated by radiography, histology, and whole bone (torsion) tests of mechanical integrity, the larger dose resulted in functional repair of the 5-mm defect beginning 4.5 weeks after surgery. The lower dose resulted in radiographic and histological evidence of new bone formation, but functional union was not observed even after 9 weeks post surgery. There was also no evidence of bone formation in control animals at this time.

Chen *et al.*, showed that a single application of 25-100 mg of recombinant TGF-β1 adjacent to cartilage induced endochondral bone formation in the rabbit ear full thickness skin wounds (Chen *et al.*, 1991). Bone formation began 21 days following the creation of the wound and reached a peak at day 42, as demonstrated by morphological methods. Active bone remodeling was observed beyond this point.

In a related study, Beck *et al.*, demonstrated that a single application of TGF-β1 in a 3% methylcellulose gel was able to repair surgically induced large skull defects that otherwise heal by fibrous connective tissue and never form bone (Beck *et al.*, 1991). Bony closure was achieved within 28 days of the application of 200 mg of

TGF-β1 and the rate of healing was shown to be dose dependent.

Studies such as those described above have thus established that exogenous growth factors can be used to stimulate new bone formation/repair/regeneration *in vivo*. Certain U.S. Patents also concern methods for treating bone defects or inducing bone formation. For example, U.S. Patent 4,877,864 relates to the administration of a therapeutic composition of bone inductive protein to treat cartilage and/or bone defects; U.S. Patent 5,108,753 concerns the use of a device containing a pure osteogenic protein to induce endochondral bone formation and for use in periodontal, dental or craniofacial reconstructive procedures.

However, nowhere in this extensive literature does there appear to be any suggestion that osteogenic genes themselves may be applied to an animal in order to promote bone repair or regeneration. Indeed, even throughout the patent literature that concerns genes encoding various bone stimulatory factors and their *in vitro* expression in host cells to produce recombinant proteins, there seems to be no mention of the possibility of using nucleic acid transfer in an effort to express an osteogenic gene in bone progenitor cells *in vivo* or to promote new bone formation in an animal or human subject.

7. Biocompatible Matrices for use in Bone Repair

There is a considerable amount of work that has been directed to the development of biocompatible matrices for use in medical implants, including those specifically for bone implantation work. In context of the present invention, a matrix may be employed in association with the gene or DNA coding region encoding the osteotropic polypeptide in order to easily deliver the gene to the

site of bone damage. Such matrices may be formed from a variety of materials presently in use for implanted medical applications.

In certain cases, the matrix may also act as a "biofiller" to provide a structure for the developing bone and cartilage. However, the formation of such a scaffolding structure is not a primary requirement, rather, the main requirements of the matrix are to be biocompatible and to be capable of delivering a nucleic acid segment to a bone cell or bone tissue site.

Matrices that may be used in certain embodiments include non-biodegradable and chemically defined matrices, such as sintered hydroxyapatite, bioglass, aluminates, and other ceramics. The bioceramics may be altered in composition, such as in calcium-aluminate-phosphate; and they may be processed to modify particular physical and chemical characteristics, such as pore size, particle size, particle shape, and biodegradability. Certain polymeric matrices may also be employed if desired, these include acrylic ester polymers and lactic acid polymers, as disclosed in U.S. Patents 4,526,909, and 4,563,489, respectively, each incorporated herein by reference. Particular examples of useful polymers are those of orthoesters, anhydrides, propylene-cofumarates, or a polymer of one or more α-hydroxy carboxylic acid monomers, *e.g.*, α-hydroxy acetic acid (glycolic acid) and/or α-hydroxy propionic acid (lactic acid).

Some of the preferred matrices for use in present purposes are those that are capable of being resorbed into the body. Potential biodegradable matrices for use in bone gene transfer include, for example, PLGA block copolymers, biodegradable and chemically defined calcium sulfate, tricalciumphosphate, hydroxyapatite, and polyanhydrides. Furthermore, biomatrices comprised of

pure proteins and/or extracellular matrix components may be employed.

The inventors have shown the use of bone or dermal collagenous materials as matrices, as may be prepared from various commercially-available lyophilized collagen preparations, such as those from bovine or rat skin, as well as PLGA block copolymers. Collagen matrices may also be formulated as described in U.S. Patent 4,394,370, incorporated herein by reference, which concerns the use of collagenous matrices as delivery vehicles for osteogenic protein. UltraFiber™, as may be obtained from Norian Corp. (Mountain View, CA), is a preferred matrix. Preferred matrices are those formulated with type II collagen, and most preferably, recombinant type II collagen and mineralized type II collagen.

Further suitable matrices may also be prepared from combinations of materials, such as PLGA block copolymers, which allow for sustained release; hydroxyapatite; or collagen and tricalciumphosphate. Although sufficient sequestration and subsequent delivery of an osteotropic gene is in no way a limitation of the present invention, should it be desired, a porous matrix and gene combination may also be administered to the bone tissue site in combination with an autologous blood clot. The basis for this is that blood clots have previously been employed to increase sequestration of osteogenic proteins for use in bone treatment (U.S. Patent 5,171,579, incorporated herein by reference) and their use in connection with the present invention is by no means excluded (they may even attract growth factors for cytokines).

8. **Collagen**

Although not previously proposed for use with a nucleic acid molecule, the use of collagen as a pharmaceutical delivery vehicle has been described. The biocompatibility of collagen matrices is well known in the art. U.S. Patents 5,206,028, 5,128,136, 5,081,106, 4,585,797, 4,390,519, and 5,197,977 (all incorporated herein by reference) describe the biocompatibility of collagen-containing matrices in the treatment of skin lesions, use as a wound dressing, and as a means of controlling bleeding. In light of these documents, therefore, there is no question concerning the suitability of applying a collagen preparation to a tissue site of an animal.

U.S. Patent 5,197,977 describes the preparation of a collagen-impregnated vascular graft including drug materials complexed with the collagen to be released slowly from the graft following implant. U.S. Patent 4,538,603 is directed to an occlusive dressing useful for treating skin lesions and a granular material capable of interacting with wound exudate. U.S. Patent 5,162,430 describes a pharmaceutically acceptable, non-immunogenic composition comprising a telopeptide collagen chemically conjugated to a synthetic hydrophilic polymer.

Further documents that one of skill in the art may find useful include U. S. Patents 4,837,285, 4,703,108, 4,409,332, and 4,347,234, each incorporated herein by reference. These references describe the uses of collagen as a non-immunogenic, biodegradable, and bioresorbable binding agent.

The inventors contemplate that collagen from many sources will be useful in the present invention. Particularly useful are the amino acid sequences of type

II collagen. Examples of type II collagen are well known in the art. For example, the amino acid sequences of human (Lee *et al.*, 1989), rat (Michaelson *et al.*, 1994), and murine (Ortman *et al.*, 1994) have been determined (SEQ ID NO:10, SEQ ID NO:12, and SEQ ID NO:14, respectively).

Although not previously known to be capable of stimulating bone progenitor cells itself, type II collagen is herein surprisingly shown to possess this property, which thus gives rise to new possibilities for clinical uses.

9. Nucleic Acid Delivery

The transfer of nucleic acids to mammalian cells has been proposed a method for treating certain diseases or disorders. Nucleic acid transfer or delivery is often referred to as "gene therapy". Initial efforts toward postnatal (somatic) gene therapy relied on indirect means of introducing genes into tissues, *e.g.*, target cells were removed from the body, infected with viral vectors carrying recombinant genes, and implanted into the body. These type of techniques are generally referred to as *ex vivo* treatment protocols. Direct *in vivo* gene transfer has recently been achieved with formulations of DNA trapped in liposomes (Ledley *et al.*, 1987); or in proteoliposomes that contain viral envelope receptor proteins (Nicolau *et al.*, 1983); calcium phosphate-coprecipitated DNA (Benvenisty and Reshef, 1986); and DNA coupled to a polylysine-glycoprotein carrier complex (Wu and Wu, 1988). The use of recombinant replication-defective viral vectors to infect target cells *in vivo* has also been described (*e.g.*, Seeger *et al.*, 1984).

In recent years, Wolff *et al.*, demonstrated that direct injection of purified preparations of DNA and RNA

into murine skeletal muscle resulted in significant reporter gene expression (Wolff *et al.*, 1990). This was an unexpected finding, and the mechanism of gene transfer could not be defined. The authors speculated that muscle cells may be particularly suited to take up and express polynucleotides *in vivo* or that damage associated with DNA injection may allow transfection to occur.

Wolff *et al.*, suggested several potential applications of the direct injection method, including (a) the treatment of heritable disorders of muscle, (b) the modification of non-muscle disorders through muscle tissue expression of therapeutic transgenes, (c) vaccine development, and (d) a reversible type of gene transfer, in which DNA is administered much like a conventional pharmaceutical treatment. In an elegant study Liu and coworkers recently showed that the direct injection method can be successfully applied to the problem of influenza vaccine development (Ulmer *et al.*, 1993).

The use of gene transfer to synoviocytes as a means of treating arthritis has also been discussed (Bandara *et al.*, 1992; Roessler *et al.*, 1993). The protocols considered have included both the *ex vivo* treatment of isolated synoviocytes and their re-introduction into the animal and also direct gene transfer in which suitable vectors are injected into the joint. The transfer of marker genes into synoviocytes has already been demonstrated using both retroviral and adenoviral technology (Bandara *et al.*, 1992; Roessler *et al.*, 1993).

Despite the exclusive emphasis on protein treatment by those working in the field of new bone growth, the present inventors saw that there was great potential for using nucleic acids themselves to promote bone regeneration/repair *in vivo*. This provides for a more sophisticated type of pharmaceutical delivery. In

addition to the ease and cost of preparing DNA, it was also reasoned that using DNA transfer rather than peptide transfer would provide many further advantages. For example, DNA transfer allows for the expression or over-expression of integral membrane receptors on the surface of bone regeneration/repair cells, whereas this cannot be done using peptide transfer because the latter (*a priori*) is an extracellular manipulation. Importantly, DNA transfer also allows for the expression of polypeptides modified in a site-directed fashion with the very minimum of additional work (*i.e.*, straightforward molecular biological manipulation without protein purification) as well as sustained release of therapies delivered by an injectable route.

The advantages of using DNA are also manifold regarding the development of pharmaceutical products and effective means of delivery. Here, important advantages include the ability to prepare injectable formulations, especially those compositions that exhibit reversible thermal gelation, and the opportunity to combine such injectables with imaging technology during delivery. "Sustained release" is also an important advantage of using DNA, in that the exogenously added DNA continues to direct the production of a protein product following incorporation into a cell. The use of certain matrix-DNA compositions also allows for a more typical "sustained release" phenomenon in that the operative release of DNA from the matrix admixture can also be manipulated.

The inventors contemplated that both naked DNA and viral-mediate DNA could be employed in an effort to transfer genes to bone progenitor cells. In beginning to study this, the most appropriate animal model had to be employed, that is, one in which the possibilities of using nucleic acids to promote bone repair could be adequately tested in controlled studies.

10. **Osteotomy Model**

Prior to the present invention, three model systems were available for study in this area, including Mov13 mice, an animal model of OI. Unfortunately, each of the models suffers from significant drawbacks. With the Mov13 mice, first, these mice typically die in young adulthood because of retrovirus-induced leukemia (Schnieke *et al.*, 1983); second, gene transfer studies in Mov13 mice conducted between postnatal weeks 8-16 (*i.e.*, prior to the development of leukemia) may be complicated by a natural adaptation in which a significant amount of new bone is deposited on the periosteal surface (Bonadio *et al.*, 1993); and third, an osteotropic gene transferred into an osteotomy site may synergize with the active retrovirus and make it even more virulent.

Another system is the *in vivo* bone fracture model created by Einhorn and colleagues (Bonnarens and Einhorn, 1984). However, this model is a closed system that would not easily permit initial studies of gene transfer *in vivo*. The organ culture model developed by Bolander and colleagues (Joyce *et al.*, 1990) was also available, but again, this model is not suitable for studying gene transfer *in vivo*. Due to the unsuitability of the above models for studying the effects of gene transfer on bone repair and regeneration, the inventors employed a rat osteotomy system, as described below.

The important features of the rat osteotomy model are as follows: under general anesthesia, four 1.2 mm diameter pins are screwed into the femoral diaphysis of normal adult Sprague-Dawley rats. A surgical template ensures parallel placement of the pins. An external fixator is then secured on the pins, and a 2 mm, or 5 mm, segmental defect is created in the central diaphysis with a Hall micro 100 oscillating saw. A biodegradable

implant material, soaked in a solution of plasmid DNA, other genetic construct or recombinant virus preparation, is then placed in the intramedullary canal and the defect is closed (FIG. 5A, FIG. 5B, FIG. 6A, FIG. 6B, FIG. 6C, FIG. 6D, FIG. 7A, FIG. 7B, FIG. 8A, FIG. 8B, FIG. 8C).

New bone formation can be detected as early as three weeks later in the 2 mm gap, although up to 9 weeks is generally allowed for new bone formation to occur. The fixator provided the necessary stability, and there were no limitations on animal ambulation. The surgical protocol has been successfully performed on 21/21 animals to date. None of these animals have died. Assays of new bone formation are performed after sacrifice, except plain film radiography, which is performed weekly from the time of surgery to sacrifice.

Previous studies in Sprague-Dawley rats have shown that the 5 mm osteotomy gap will heal as a fibrous non-union, whereas a gap of less than 3 mm, (such as the 2 mm gap routinely employed in the studies described herein) will heal by primary bone formation. Studies using the 5 mm gap thus allow a determination of whether transgene expression can stimulate new bone formation when fibrous tissue healing normally is expected. On the other hand, studies with the 2 mm gap allow a determination of whether transgene expression can speed up natural primary bone healing. Controls were also performed in which animals received no DNA (FIG. 9A and FIG. 9B).

11. Gene Transfer Promotes Bone Repair *In Vivo*

The present inventors surprisingly found that gene transfer into bone progenitor cells *in vivo* (*i.e.*, cells in the regenerating tissue in the osteotomy gap) could be readily achieved. Currently, the preferred methods for achieving gene transfer generally involve using a fibrous

collagen implant material soaked in a solution of DNA shortly before being placed in the site in which one desires to promote bone growth. As the studies presented herein show, the implant material facilitates the uptake of exogenous plasmid constructs by cells (in the osteotomy gap) which clearly participate in bone regeneration/repair. The transgenes, following cellular uptake, direct the expression of recombinant polypeptides, as evidenced by the *in vivo* expression of functional marker gene products.

Further studies are presented herein demonstrating that the transfer of an osteotropic gene results in cellular expression of a recombinant osteotropic molecule, which expression is directly associated with stimulation of new bone formation. After considering a relatively large number of candidate genes, a gene transfer vector coding for a fragment of human parathyroid hormone (hPTH1-34) was chosen for the inventors' initial studies. Several factors were considered in making this choice: (a), recombinant hPTH1-34 peptides can be discriminated from any endogenous rat hormone present in osteotomy tissues; (b), hPTH1-34 peptides will stimulate new bone formation in Sprague-Dawley rats, indicating that the human peptide can efficiently bind the PTH/PTHrP receptor on the rat osteoblast cell surface; and (c), there is only one PTH/PTHrP receptor, the gene for this receptor has been cloned, and cDNA probes to the receptor are available.

Thus, in terms of understanding the mechanism of action of the transgene on new bone formation *in vivo*, the inventors reasoned it most straightforward to correlate the expression of recombinant hPTH1-34 peptide and its receptor with new bone formation in the rat osteotomy model. Of course, following these initial studies, it is contemplated that any one of a wide

variety of genes may be employed in connection with the bone gene transfer embodiments of the present invention.

Previous studies have indicated that hPTH1-34 is a more powerful anabolic agent when given intermittently as opposed to continuously. Despite the fact that an anabolic effect would still be expected with continuous dosing, as documented by the studies of Parsons and co-workers (Tam *et al.*, 1982; Spencer *et al.*, 1989), there was a concern that the PLJ-hPTH1-34 transgene may not function very effectively as transfected cells would be expected to express recombinant hPTH1-34 molecules in a constitutive manner. The finding that transfection and expression of the LPH-hPTH1-34 transgene did effectively stimulate bone formation in the rat osteotomy model was therefore an important result.

As the osteotomy site in this model is highly vascularized, one possible complication of the studies with the PLJ-hPTH1-34 transgene is the secretion of recombinant human PTH from the osteotomy site with consequent hypercalcemia and (potentially) animal death. Weekly serum calcium levels should therefore be determined when using this transgene. The fact that no evidence of disturbed serum calcium levels has been found in this work is therefore a further encouraging finding.

These studies complement others by the inventors in which direct gene transfer was employed to introduce genes into Achilles' tendon and cruciate ligament, as described in Example XI.

Various immediate applications for using nucleic acid delivery in connection with bone disorders became apparent to the inventors following these surprising findings. The direct transfer of an osteotropic gene to promote fracture repair in clinical orthopaedic practice

is just one use. Other important aspects of this technology include the use of gene transfer to treat patients with "weak bones", such as in diseases like osteoporosis; to improve poor healing which may arise for unknown reasons, *e.g.*, fibrous non-union; to promote implant integration and the function of artificial joints; to stimulate healing of other skeletal tissues such as Achilles' tendon; and as an adjuvant to repair large defects. In all such embodiments, DNA is being used as a direct pharmaceutical agent.

12. Biological Functional Equivalents

As mentioned above, modification and changes may be made in the structure of an osteotropic gene and still obtain a functional molecule that encodes a protein or polypeptide with desirable characteristics. The following is a discussion based upon changing the amino acids of a protein to create an equivalent, or even an improved, second-generation molecule. The amino acid changes may be achieved by changing the codons of the DNA sequence, according to the following codon table:

Table 1

Amino Acids			Codons					
Alanine	Ala	A	GCA	GCC	GCG	GCU		
Cysteine	Cys	C	UGC	UGU				
Aspartic acid	Asp	D	GAC	GAU				
Glutamic acid	Glu	E	GAA	GAG				
Phenylalanine	Phe	F	UUC	UUU				
Glycine	Gly	G	GGA	GGC	GGG	GGU		
Histidine	His	H	CAC	CAU				
Isoleucine	Ile	I	AUA	AUC	AUU			
Lysine	Lys	K	AAA	AAG				
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU
Methionine	Met	M	AUG					
Asparagine	Asn	N	AAC	AAU				
Proline	Pro	P	CCA	CCC	CCG	CCU		
Glutamine	Gln	Q	CAA	CAG				
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	UCU
Threonine	Thr	T	ACA	ACC	ACG	ACU		
Valine	Val	V	GUA	GUC	GUG	GUU		
Tryptophan	Trp	W	UGG					
Tyrosine	Tyr	Y	UAC	UAU				

For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies or binding sites on substrate molecules. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid sequence substitutions can be made in a protein sequence, and, of course, its underlying DNA coding sequence, and nevertheless obtain a protein with like properties. It is thus contemplated by the inventors that various changes may be made in the DNA sequences of osteotropic genes without appreciable loss of their biological utility or activity.

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte and Doolittle, 1982, incorporate herein by reference). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics (Kyte and Doolittle, 1982), these are: Isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, *i.e.*, still obtain a biological functionally equivalent protein. In making such changes, the substitution of amino acids whose hydropathic indices are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U.S. Patent 4,554,101, incorporated herein by reference, states that the

greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein.

As detailed in U.S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 ± 1); alanine (-0.5); histidine *-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent, and in particular, an immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

As outlined above, amino acid substitutions are generally therefore based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions which take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

13. **Site-Specific Mutagenesis**

Site-specific mutagenesis is a technique useful in the preparation of individual peptides, or biologically functional equivalent proteins or peptides, through specific mutagenesis of the underlying DNA. The technique further provides a ready ability to prepare and test sequence variants, for example, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 17 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered.

In general, the technique of site-specific mutagenesis is well known in the art, as exemplified by various publications. As will be appreciated, the technique typically employs a phage vector which exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage are readily commercially available and their use is generally well known to those skilled in the art. Double stranded plasmids are also routinely employed in site directed mutagenesis which eliminates the step of transferring the gene of interest from a plasmid to a phage.

In general, site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded vector or melting apart of two strands of a

double stranded vector which includes within its sequence a DNA sequence which encodes the desired osteotropic protein. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically. This primer is then annealed with the single-stranded vector, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as *E. coli* cells, and clones are selected which include recombinant vectors bearing the mutated sequence arrangement.

The preparation of sequence variants of the selected osteotropic gene using site-directed mutagenesis is provided as a means of producing potentially useful species and is not meant to be limiting as there are other ways in which sequence variants of osteotropic genes may be obtained. For example, recombinant vectors encoding the desired osteotropic gene may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants.

14. Monoclonal Antibody Generation

Means for preparing and characterizing antibodies are well known in the art (See, *e.g.*, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, 1988; incorporated herein by reference).

The methods for generating monoclonal antibodies (MAbs) generally begin along the same lines as those for preparing polyclonal antibodies. Briefly, a polyclonal antibody is prepared by immunizing an animal with an immunogenic composition in accordance with the present invention and collecting antisera from that immunized animal. A wide range of animal species can be used for the production of antisera. Typically the animal used for production of anti-antisera is a rabbit, a mouse, a rat, a hamster, a guinea pig or a goat. Because of the relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

As is well known in the art, a given composition may vary in its immunogenicity. It is often necessary therefore to boost the host immune system, as may be achieved by coupling a peptide or polypeptide immunogen to a carrier. Exemplary and preferred carriers are keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Other albumins such as ovalbumin, mouse serum albumin or rabbit serum albumin can also be used as carriers. Means for conjugating a polypeptide to a carrier protein are well known in the art and include glutaraldehyde, m-maleimidobencoyl-N-hydroxysuccinimide ester, carbodiimyde and bis-biazotized benzidine.

As is also well known in the art, the immunogenicity of a particular immunogen composition can be enhanced by the use of non-specific stimulators of the immune response, known as adjuvants. Exemplary and preferred

adjuvants include complete Freund's adjuvant (a non-specific stimulator of the immune response containing killed *Mycobacterium tuberculosis)*, incomplete Freund's adjuvants and aluminum hydroxide adjuvant.

The amount of immunogen composition used in the production of polyclonal antibodies varies upon the nature of the immunogen as well as the animal used for immunization. A variety of routes can be used to administer the immunogen (subcutaneous, intramuscular, intradermal, intravenous and intraperitoneal). The production of polyclonal antibodies may be monitored by sampling blood of the immunized animal at various points following immunization. A second, booster injection, may also be given. The process of boosting and titering is repeated until a suitable titer is achieved. When a desired level of immunogenicity is obtained, the immunized animal can be bled and the serum isolated and stored, and/or the animal can be used to generate MAbs.

MAbs may be readily prepared through use of well-known techniques, such as those exemplified in U.S. Patent 4,196,265, incorporated herein by reference. Typically, this technique involves immunizing a suitable animal with a selected immunogen composition, e.g., a purified or partially purified LTBP-3 protein, polypeptide or peptide. The immunizing composition is administered in a manner effective to stimulate antibody producing cells. Rodents such as mice and rats are preferred animals, however, the use of rabbit, sheep frog cells is also possible. The use of rats may provide certain advantages (Goding, 1986, pp. 60-61), but mice are preferred, with the BALB/c mouse being most preferred

as this is most routinely used and generally gives a higher percentage of stable fusions.

Following immunization, somatic cells with the potential for producing antibodies, specifically B lymphocytes (B cells), are selected for use in the MAb generating protocol. These cells may be obtained from biopsied spleens, tonsils or lymph nodes, or from a peripheral blood sample. Spleen cells and peripheral blood cells are preferred, the former because they are a rich source of antibody-producing cells that are in the dividing plasmablast stage, and the latter because peripheral blood is easily accessible. Often, a panel of animals will have been immunized and the spleen of animal with the highest antibody titer will be removed and the spleen lymphocytes obtained by homogenizing the spleen with a syringe. Typically, a spleen from an immunized mouse contains approximately 5 X 10^7 to 2 X 10^8 lymphocytes.

The antibody-producing B lymphocytes from the immunized animal are then fused with cells of an immortal myeloma cell, generally one of the same species as the animal that was immunized. Myeloma cell lines suited for use in hybridoma-producing fusion procedures preferably are non-antibody-producing, have high fusion efficiency, and enzyme deficiencies that render then incapable of growing in certain selective media which support the growth of only the desired fused cells (hybridomas).

Any one of a number of myeloma cells may be used, as are known to those of skill in the art (Goding, pp. 65-66, 1986; Campbell, pp. 75-83, 1984). For example, where the immunized animal is a mouse, one may use P3-X63/Ag8, X63-Ag8.653, NS1/1.Ag 4 1, Sp210-Ag14, FO, NSO/U, MPC-11, MPC11-X45-GTG 1.7 and S194/5XX0 Bul; for rats, one may use R210.RCY3, Y3-Ag 1.2.3, IR983F and

4B210; and U-266, GM1500-GRG2, LICR-LON-HMy2 and UC729-6 are all useful in connection with human cell fusions.

One preferred murine myeloma cell is the NS-1 myeloma cell line (also termed P3-NS-1-Ag4-1), which is readily available from the NIGMS Human Genetic Mutant Cell Repository by requesting cell line repository number GM3573. Another mouse myeloma cell line that may be used is the 8-azaguanine-resistant mouse murine myeloma SP2/0 non-producer cell line.

Methods for generating hybrids of antibody-producing spleen or lymph node cells and myeloma cells usually comprise mixing somatic cells with myeloma cells in a 2:1 proportion, though the proportion may vary from about 20:1 to about 1:1, respectively, in the presence of an agent or agents (chemical or electrical) that promote the fusion of cell membranes. Fusion methods using Sendai virus have been described by Kohler and Milstein (1975; 1976), and those using polyethylene glycol (PEG), such as 37% (v/v) PEG, by Gefter *et al.* (1977). The use of electrically induced fusion methods is also appropriate (Goding pp. 71-74, 1986).

Fusion procedures usually produce viable hybrids at low frequencies, about 1×10^{-6} to 1×10^{-8}. However, this does not pose a problem, as the viable, fused hybrids are differentiated from the parental, unfused cells (particularly the unfused myeloma cells that would normally continue to divide indefinitely) by culturing in a selective medium. The selective medium is generally one that contains an agent that blocks the *de novo* synthesis of nucleotides in the tissue culture media. Exemplary and preferred agents are aminopterin, methotrexate, and azaserine. Aminopterin and methotrexate block *de novo* synthesis of both purines and pyrimidines, whereas azaserine blocks only purine

synthesis. Where aminopterin or methotrexate is used, the media is supplemented with hypoxanthine and thymidine as a source of nucleotides (HAT medium). Where azaserine is used, the media is supplemented with hypoxanthine.

The preferred selection medium is HAT. Only cells capable of operating nucleotide salvage pathways are able to survive in HAT medium. The myeloma cells are defective in key enzymes of the salvage pathway, *e.g.*, hypoxanthine phosphoribosyl transferase (HPRT), and they cannot survive. The B cells can operate this pathway, but they have a limited life span in culture and generally die within about two weeks. Therefore, the only cells that can survive in the selective media are those hybrids formed from myeloma and B cells.

This culturing provides a population of hybridomas from which specific hybridomas are selected. Typically, selection of hybridomas is performed by culturing the cells by single-clone dilution in microtiter plates, followed by testing the individual clonal supernatants (after about two to three weeks) for the desired reactivity. The assay should be sensitive, simple and rapid, such as radioimmunoassays, enzyme immunoassays, cytotoxicity assays, plaque assays, dot immunobinding assays, and the like.

The selected hybridomas would then be serially diluted and cloned into individual antibody-producing cell lines, which clones can then be propagated indefinitely to provide MAbs. The cell lines may be exploited for MAb production in two basic ways. A sample of the hybridoma can be injected (often into the peritoneal cavity) into a histocompatible animal of the type that was used to provide the somatic and myeloma cells for the original fusion. The injected animal develops tumors secreting the specific monoclonal

antibody produced by the fused cell hybrid. The body fluids of the animal, such as serum or ascites fluid, can then be tapped to provide MAbs in high concentration. The individual cell lines could also be cultured *in vitro*, where the MAbs are naturally secreted into the culture medium from which they can be readily obtained in high concentrations. MAbs produced by either means may be further purified, if desired, using filtration, centrifugation and various chromatographic methods such as HPLC or affinity chromatography.

15. LTBP-3

Other aspects of the present invention concern isolated DNA segments and recombinant vectors encoding LTBP-3, and the creation and use of recombinant host cells through the application of DNA technology, that express LTBP-3 gene products. As such, the invention concerns DNA segment comprising an isolated gene that encodes a protein or peptide that includes an amino acid sequence essentially as set forth by a contiguous sequence from SEQ ID NO:3. These DNA segments are represented by those that include a nucleic acid sequence essentially as set forth by a contiguous sequence from SEQ ID NO:2 (FIG. 25). Compositions that include a purified protein that has an amino acid sequence essentially as set forth by the amino acid sequence of SEQ ID NO:3 (FIG. 26) are also encompassed by the invention.

The TGF-βs represent a family of structurally related molecules with diverse effects on mammalian cell shape, growth, and differentiation (Roberts and Sporn, 1990). Initially synthesized as a precursor consisting of an amino-terminal propeptide followed by mature TGF-β, two chains of nascent pro-TGF-β associate in most tissues to form a Mr ~106,000 inactive disulfide-bonded dimer.

Homodimers are most common, but heterodimers have also been described (Cheifetz *et al.*, 1987; Ogawa *et al.*, 1992). During biosynthesis the mature TGF-β dimer is cleaved from the propeptide dimer. TGF-β latency results in part from the non-covalent association of propeptide and mature TGF-β dimers (Pircher *et al.*, 1984, 1986; Wakefield *et al.*, 1987; Millan *et al.*, 1992; Miyazono and Heldin, 1989). Consequently, the propeptide dimer is often referred to as the latency associated protein (LAP), and LAP plus the disulfide-bonded TGF-β dimer are also known as the small latent complex. In the extracellular space small latent complexes must be dissociated to activate mature TGF-β. The mechanism of activation of the latent complex is thought to be one of the most important steps governing TGF-β effects (Lyons *et al.*, 1988; Antonelli-Orlidge *et al.*, 1989; Twardzik *et al.*, 1990; Sato *et al.*, 1993).

In certain lines of cultured cells small latent growth factor complexes may contain additional high molecular weight proteins. The best characterized of these high molecular weight proteins is the latent TGF-β binding protein, or LTBP (Miyazono *et al.*, 1988; Kanzaki *et al.*, 1990; Tsuji *et al.*, 1990; Olofsson *et al.*, 1992; Taketazu *et al.*, 1994). LTBP produced by different cell types is heterogeneous in size, perhaps because of alternative splicing or because of tissue-specific proteolytic processing (Miyazono *et al.*, 1988; Wakefield *et al.*, 1988; Kanzaki *et al.*, 1990; Tsuji *et al.*, 1990). Latent TGF-β complexes that contain LTBP are known as large latent complexes. LTBP has no known covalent linkage to mature TGF-β, but rather it is linked by a disulfide bond to LAP.

Regarding the novel protein LTBP-3, the present invention concerns DNA segments, that can be isolated from virtually any mammalian source, that are free from total genomic DNA and that encode proteins having LTBP-3-like activity. DNA segments encoding LTBP-3-like species may prove to encode proteins, polypeptides, subunits, functional domains, and the like.

As used herein, the term "DNA segment" refers to a DNA molecule that has been isolated free of total genomic DNA of a particular species. Therefore, a DNA segment encoding LTBP-3 refers to a DNA segment that contains LTBP-3 coding sequences yet is isolated away from, or purified free from, total genomic DNA of the species from which the DNA segment is obtained. Included within the term "DNA segment", are DNA segments and smaller fragments of such segments, and also recombinant vectors, including, for example, plasmids, cosmids, phagemids, phage, viruses, and the like.

Similarly, a DNA segment comprising an isolated or purified LTBP-3 gene refers to a DNA segment including LTBP-3 coding sequences and, in certain aspects, regulatory sequences, isolated substantially away from other naturally occurring genes or protein encoding sequences. In this respect, the term "gene" is used for simplicity to refer to a functional protein, polypeptide or peptide encoding unit. As will be understood by those in the art, this functional term includes both genomic sequences, cDNA sequences and smaller engineered gene segments that express, or may be adapted to express, proteins, polypeptides or peptides.

"Isolated substantially away from other coding sequences" means that the gene of interest, in this case, a gene encoding LTBP-3, forms the significant part of the

coding region of the DNA segment, and that the DNA segment does not contain large portions of naturally-occurring coding DNA, such as large chromosomal fragments or other functional genes or cDNA coding regions. Of course, this refers to the DNA segment as originally isolated, and does not exclude genes or coding regions later added to the segment by the hand of man.

In particular embodiments, the invention concerns isolated DNA segments and recombinant vectors incorporating DNA sequences that encode an LTBP-3 species that includes within its amino acid sequence an amino acid sequence essentially as set forth in SEQ ID NO:3. In other particular embodiments, the invention concerns isolated DNA segments and recombinant vectors incorporating DNA sequences that include within their sequence a nucleotide sequence essentially as set forth in SEQ ID NO:2.

The term "a sequence essentially as set forth in SEQ ID NO:3" means that the sequence substantially corresponds to a portion of SEQ ID NO:3 and has relatively few amino acids that are not identical to, or a biologically functional equivalent of, the amino acids of SEQ ID NO:3. The term "biologically functional equivalent" is well understood in the art and is further defined in detail herein (for example, see section 7, preferred embodiments). Accordingly, sequences that have between about 70% and about 80%; or more preferably, between about 81% and about 90%; or even more preferably, between about 91% and about 99%; of amino acids that are identical or functionally equivalent to the amino acids of SEQ ID NO:3 will be sequences that are "essentially as set forth in SEQ ID NO:3".

In certain other embodiments, the invention concerns isolated DNA segments and recombinant vectors that

include within their sequence a nucleic acid sequence essentially as set forth in SEQ ID NO:2. The term "essentially as set forth in SEQ ID NO:2" is used in the same sense as described above and means that the nucleic acid sequence substantially corresponds to a portion of SEQ ID NO:2 and has relatively few codons that are not identical, or functionally equivalent, to the codons of SEQ ID NO:2. Again, DNA segments that encode proteins exhibiting LTBP-3-like activity will be most preferred.

It will also be understood that amino acid and nucleic acid sequences may include additional residues, such as additional N- or C-terminal amino acids or 5' or 3' sequences, and yet still be essentially as set forth in one of the sequences disclosed herein, so long as the sequence meets the criteria set forth above, including the maintenance of biological protein activity where protein expression is concerned. The addition of terminal sequences particularly applies to nucleic acid sequences that may, for example, include various non-coding sequences flanking either of the 5' or 3' portions of the coding region or may include various internal sequences, *i.e.*, introns, which are known to occur within genes.

Naturally, the present invention also encompasses DNA segments that are complementary, or essentially complementary, to the sequence set forth in SEQ ID NO:2. Nucleic acid sequences that are "complementary" are those that are capable of base-pairing according to the standard Watson-Crick complementarity rules. As used herein, the term "complementary sequences" means nucleic acid sequences that are substantially complementary, as may be assessed by the same nucleotide comparison set forth above, or as defined as being capable of hybridizing to the nucleic acid segment of SEQ ID NO:2,

under relatively stringent conditions such as those described herein.

The nucleic acid segments of the present invention, regardless of the length of the coding sequence itself, may be combined with other DNA sequences, such as promoters, polyadenylation signals, additional restriction enzyme sites, multiple cloning sites, other coding segments, and the like, such that their overall length may vary considerably. It is therefore contemplated that a nucleic acid fragment of almost any length may be employed, with the total length preferably being limited by the ease of preparation and use in the intended recombinant DNA protocol. For example, nucleic acid fragments may be prepared that include a short contiguous stretch identical to or complementary to SEQ ID NO:2, such as about 14 nucleotides, and that are up to about 10,000 or about 5,000 base pairs in length, with segments of about 3,000 being preferred in certain cases. DNA segments with total lengths of about 1,000, about 500, about 200, about 100 and about 50 base pairs in length (including all intermediate lengths) are also contemplated to be useful.

It will be readily understood that "intermediate lengths", in these contexts, means any length between the quoted ranges, such as 14, 15, 16, 17, 18, 19, 20, *etc.*; 21, 22, 23, *etc.*; 30, 31, 32, *etc.*; 50, 51, 52, 53, *etc.*; 100, 101, 102, 103, *etc.*; 150, 151, 152, 153, *etc.*; including all integers through the 200-500; 500-1,000; 1,000-2,000; 2,000-3,000; 3,000-5,000; 5,000-10,000 ranges, up to and including sequences of about 12,001, 12,002, 13,001, 13,002 and the like.

It will also be understood that this invention is not limited to the particular nucleic acid and amino acid sequences of SEQ ID NO:2 and SEQ ID NO:3. Recombinant

vectors and isolated DNA segments may therefore variously include the LTBP-3 coding regions themselves, coding regions bearing selected alterations or modifications in the basic coding region, or they may encode larger polypeptides that nevertheless include LTBP-3-coding regions or may encode biologically functional equivalent proteins or peptides that have variant amino acids sequences.

The DNA segments of the present invention encompass biologically functional equivalent LTBP-3 proteins and peptides. Such sequences may arise as a consequence of codon redundancy and functional equivalency that are known to occur naturally within nucleic acid sequences and the proteins thus encoded. Alternatively, functionally equivalent proteins or peptides may be created via the application of recombinant DNA technology, in which changes in the protein structure may be engineered, based on considerations of the properties of the amino acids being exchanged. Changes designed by man may be introduced through the application of site-directed mutagenesis techniques, *e.g.*, to introduce improvements to the antigenicity of the protein or to test mutants in order to examine activity at the molecular level.

If desired, one may also prepare fusion proteins and peptides, *e.g.*, where the LTBP-3 coding regions are aligned within the same expression unit with other proteins or peptides having desired functions, such as for purification or immunodetection purposes (*e.g.*, proteins that may be purified by affinity chromatography and enzyme label coding regions, respectively).

Recombinant vectors form further aspects of the present invention. Particularly useful vectors are contemplated to be those vectors in which the coding

portion of the DNA segment, whether encoding a full length protein or smaller peptide, is positioned under the control of a promoter. The promoter may be in the form of the promoter that is naturally associated with a LTBP-3 gene, as may be obtained by isolating the 5' non-coding sequences located upstream of the coding segment or exon, for example, using recombinant cloning and/or PCR™ technology, in connection with the compositions disclosed herein.

In other embodiments, it is contemplated that certain advantages will be gained by positioning the coding DNA segment under the control of a recombinant, or heterologous, promoter. As used herein, a recombinant or heterologous promoter is intended to refer to a promoter that is not normally associated with an LTBP-3 gene in its natural environment. Such promoters may include LTBP-3 promoters normally associated with other genes, and/or promoters isolated from any bacterial, viral, eukaryotic, or mammalian cell. Naturally, it will be important to employ a promoter that effectively directs the expression of the DNA segment in the cell type, organism, or even animal, chosen for expression. The use of promoter and cell type combinations for protein expression is generally known to those of skill in the art of molecular biology, for example, see Sambrook *et al.*, 1989. The promoters employed may be constitutive, or inducible, and can be used under the appropriate conditions to direct high level expression of the introduced DNA segment, such as is advantageous in the large-scale production of recombinant proteins or peptides. Appropriate promoter systems contemplated for use in high-level expression include, but are not limited

to, the *Pichia* expression vector system (Pharmacia LKB Biotechnology) (see Example XVI herein).

In connection with expression embodiments to prepare recombinant LTBP-3 proteins and peptides, it is contemplated that longer DNA segments will most often be used, with DNA segments encoding the entire LTBP-3 protein or functional domains, subunits, *etc.* being most preferred. However, it will be appreciated that the use of shorter DNA segments to direct the expression of LTBP-3 peptides or epitopic core regions, such as may be used to generate anti-LTBP-3 antibodies, also falls within the scope of the invention. DNA segments that encode peptide antigens from about 15 to about 50 amino acids in length, or more preferably, from about 15 to about 30 amino acids in length are contemplated to be particularly useful.

The LTBP-3 gene and DNA segments may also be used in connection with somatic expression in an animal or in the creation of a transgenic animal. Again, in such embodiments, the use of a recombinant vector that directs the expression of the full length or active LTBP-3 protein is particularly contemplated.

In addition to their use in directing the expression of the LTBP-3 protein, the nucleic acid sequences disclosed herein also have a variety of other uses. For example, they also have utility as probes or primers in nucleic acid hybridization embodiments. As such, it is contemplated that nucleic acid segments that comprise a sequence region that consists of at least a 14 nucleotide long contiguous sequence that has the same sequence as, or is complementary to, a 14 nucleotide long contiguous sequence of SEQ ID NO:2 will find particular utility. Longer contiguous identical or complementary sequences, *e.g.*, those of about 20, 30, 40, 50, 100, 200, 500, 1000

(including all intermediate lengths) and even up to full length sequences will also be of use in certain embodiments.

The ability of such nucleic acid probes to specifically hybridize to LTBP-3-encoding sequences will enable them to be of use in detecting the presence of complementary sequences in a given sample. However, other uses are envisioned, including the use of the sequence information for the preparation of mutant species primers, or primers for use in preparing other genetic constructions.

Nucleic acid molecules having sequence regions consisting of contiguous nucleotide stretches of 10-14, 15-20, 30, 50, or even of 100-200 nucleotides or so, identical or complementary to SEQ ID NO:2, are particularly contemplated as hybridization probes for use in, *e.g.*, Southern and Northern blotting. This would allow LTBP-3 structural or regulatory genes to be analyzed, both in diverse cell types and also in various mammalian cells. The total size of fragment, as well as the size of the complementary stretch(es), will ultimately depend on the intended use or application of the particular nucleic acid segment. Smaller fragments will generally find use in hybridization embodiments, wherein the length of the contiguous complementary region may be varied, such as between about 10-14 and about 100 nucleotides, but larger contiguous complementarity stretches may be used, according to the length complementary sequences one wishes to detect.

The use of a hybridization probe of about 10-14 nucleotides in length allows the formation of a duplex molecule that is both stable and selective. Molecules having contiguous complementary sequences over stretches greater than 10 bases in length are generally preferred,

though, in order to increase stability and selectivity of the hybrid, and thereby improve the quality and degree of specific hybrid molecules obtained. One will generally prefer to design nucleic acid molecules having gene-complementary stretches of 15 to 20 contiguous nucleotides, or even longer where desired.

Hybridization probes may be selected from any portion of any of the sequences disclosed herein. All that is required is to review the sequence set forth in SEQ ID NO:2 and to select any continuous portion of the sequence, from about 10-14 nucleotides in length up to and including the full length sequence, that one wishes to utilize as a probe or primer. The choice of probe and primer sequences may be governed by various factors, such as, by way of example only, one may wish to employ primers from towards the termini of the total sequence.

The process of selecting and preparing a nucleic acid segment that includes a contiguous sequence from within SEQ ID NO:2 may alternatively be described as preparing a nucleic acid fragment. Of course, fragments may also be obtained by other techniques such as, *e.g.*, by mechanical shearing or by restriction enzyme digestion. Small nucleic acid segments or fragments may be readily prepared by, for example, directly synthesizing the fragment by chemical means, as is commonly practiced using an automated oligonucleotide synthesizer. Also, fragments may be obtained by application of nucleic acid reproduction technology, such as the PCR™ technology of U.S. Patent 4,603,102 (incorporated herein by reference), by introducing selected sequences into recombinant vectors for recombinant production, and by other recombinant DNA techniques generally known to those of skill in the art of molecular biology.

Accordingly, the nucleotide sequences of the invention may be used for their ability to selectively form duplex molecules with complementary stretches of LTBP-3 gene or cDNA fragments. Depending on the application envisioned, one will desire to employ varying conditions of hybridization to achieve varying degrees of selectivity of probe towards target sequence. For applications requiring high selectivity, one will typically desire to employ relatively stringent conditions to form the hybrids, *e.g.*, one will select relatively low salt and/or high temperature conditions, such as provided by about 0.02 M to about 0.15 M NaCl at temperatures of 50°C to 70°C. Such selective conditions tolerate little, if any, mismatch between the probe and the template or target strand, and would be particularly suitable for isolating LTBP-3 genes.

Of course, for some applications, for example, where one desires to prepare mutants employing a mutant primer strand hybridized to an underlying template or where one seeks to isolate LTBP-3-encoding sequences from related species, functional equivalents, or the like, less stringent hybridization conditions will typically be needed in order to allow formation of the heteroduplex. In these circumstances, one may desire to employ conditions such as about 0.15 M to about 0.9 M salt, at temperatures ranging from 20°C to 55°C. Cross-hybridizing species can thereby be readily identified as positively hybridizing signals with respect to control hybridizations. In any case, it is generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide, which serves to destabilize the hybrid duplex in the same manner as increased temperature. Thus, hybridization conditions can be readily manipulated, and thus will generally be a method of choice depending on the desired results.

In certain embodiments, it will be advantageous to employ nucleic acid sequences of the present invention in combination with an appropriate means, such as a label, for determining hybridization. A wide variety of appropriate indicator means are known in the art, including fluorescent, radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of giving a detectable signal. In preferred embodiments, one will likely desire to employ a fluorescent label or an enzyme tag, such as urease, alkaline phosphatase or peroxidase, instead of radioactive or other environmental undesirable reagents. In the case of enzyme tags, colorimetric indicator substrates are known that can be employed to provide a means visible to the human eye or spectrophotometrically, to identify specific hybridization with complementary nucleic acid-containing samples.

In general, it is envisioned that the hybridization probes described herein will be useful both as reagents in solution hybridization as well as in embodiments employing a solid phase. In embodiments involving a solid phase, the test DNA (or RNA) is adsorbed or otherwise affixed to a selected matrix or surface. This fixed, single-stranded nucleic acid is then subjected to specific hybridization with selected probes under desired conditions. The selected conditions will depend on the particular circumstances based on the particular criteria required (depending, for example, on the G+C content, type of target nucleic acid, source of nucleic acid, size of hybridization probe, *etc.*). Following washing of the hybridized surface so as to remove nonspecifically bound probe molecules, specific hybridization is detected, or even quantitated, by means of the label.

The following examples are included to demonstrate preferred embodiments of the invention. It should be

appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE I

ANIMAL MODEL FOR ASSESSING NEW BONE FORMATION

As various animal models were not suitable for studying the effects of nucleic acid transfer on bone formation, the inventors employed the following model system. The important features of the rat osteotomy model are as described in the following protocol (which is generally completed in 25-35 minutes).

The osteotomy was performed on one femur per animal. Right to left differences have not been apparent, but such differences are monitored in these studies, since the limb receiving the osteotomy is randomized.

After pre-operative preparation (*i.e.*, shaving and Betadine® scrub), adult male Sprague Dawley rats (~500 gm, retired male breeders) were anesthetized using a 3% halothane 97% oxygen mixture (700 ml/min. flow rate). A lateral approach to the femur was made on one limb. Utilizing specially designed surgical guides, four 1.2-mm diameter pins were screwed into the diaphysis after pre-drilling with a high speed precision bit. A surgical template ensured precise and parallel placement of the pins. The order of pin placement was always the same:

outer proximal first and then outer distal, inner proximal and inner distal (with "outer" and "inner" referring to the distance from the hip joint). Pin placement in the center of the femur was ensured by fluoroscopic imaging during pin placement. The external fixator was secured on the pins and a t mm or 2 mm segmental defect was created in the central diaphysis through an incision using a Hall Micro 100 Oscillating saw (#5053-60 Hall surgical blades) under constant irrigation. Other than the size of the segmental defect, there is no difference between the 5 mm and 2 mm osteotomy protocols (FIG. 5A, FIG. 5B, FIG. 6A, FIG. 6B, FIG. 6C, FIG. 6D, FIG. 7A, FIG. 7B, FIG. 8A, FIG. 8B, FIG. 8C).

The contents of the osteotomy site were irrigated with sterile saline and the fibrous collagen implant material, previously soaked in a solution of plasmid DNA or other DNA construct, if appropriate, was placed *in situ*. The wound was then closed in layers. Since the fixator provided the necessary stability no limitations on animal ambulation existed, and other supports were not required. The surgical protocol has been successfully performed on 53 animals to date, including 35 controls (Table 2 and FIG. 24). None of these animals have died and no significant adverse effects have been observed, other than complications that might be associated with surgical fracture repair. Minor complications that were experienced include 1 animal that developed a post-operative osteomyelitis and 1 animal in which 2/4 pins loosened as a consequence of post-operative bone fracture.

EXAMPLE II

IMPLANT MATERIAL FOR USE IN BONE GENE TRANSFER

Various implant materials may be used for transferring genes into the site of bone repair and/or regeneration *in vivo*. These materials are soaked in a solution containing the DNA or gene that is to be transferred to the bone regrowth site. Alternatively, DNA may be incorporated into the matrix as a preferred method of making.

One particular example of a suitable material is fibrous collagen, which may be lyophilized following extraction and partial purification from tissue and then sterilized. A particularly preferred collagen is the fibrous collagen implant material termed UltraFiber™, as may be obtained from Norian Corp., (Mountain View, CA). Detailed descriptions of the composition and use of UltraFiber™ are provided in Gunasekaran *et al.*, (1993a, b; each incorporated herein by reference).

A more particularly preferred collagen is type II collagen, with most particularly preferred collagen being either recombinant type II collagen, or mineralized type II collagen. Prior to placement in osteotomy sites, implant materials are soaked in solutions of DNA (or virus) under sterile conditions. The soaking may be for any appropriate and convenient period, *e.g.*, from 6 minutes to over-night. The DNA (*e.g.*, plasmid) solution will be a sterile aqueous solution, such as sterile water or an acceptable buffer, with the concentration generally being about 0.5 - 1.0 mg/ml. Currently preferred plasmids are those such as pGL2 (Promega), pSV40β-gal, pAd.CMV*lacZ*, and pLJ.

EXAMPLE III

PARATHYROID HORMONE GENE CONSTRUCTS

The active fragment of the human parathyroid hormone gene (hPTH1-34) was chosen as the first of the osteotropic genes to be incorporated into an expression vector for use in gene transfer to promote new bone formation in the rat osteotomy model.

The inventors chose to construct the hPTH1-34 transgene in the pLJ expression vector (FIG. 10), since this vector was appropriate for studies of transgene function both *in vitro* and *in vivo*. A schematic of the PLJ-hPTH1-34 transgene is shown in FIG. 10. The DNA and amino acid sequences of the hPTH1-34 are well known, *e.g.*, see Hendy *et al.*, (1981, incorporated herein by reference). To insert the transgene into the PLJ expression vector PCR™ of a full-length PTH recombinant clone was employed, followed by standard molecular biological manipulation.

A retroviral stock was then generated following $CaPO_4$-mediated transfection of ϕ crip cells with the PLJ-hPTH1-34 construct, all according to standard protocols (Sambrook *et al.*, 1989). Independent transduced Rat-1 clones were obtained by standard infection and selection procedures (Sambrook *et al.*, 1989).

One clone (YZ-15) was analyzed by Southern analysis, demonstrating that the PLJ-hPTH1-34 transgene had stably integrated into the Rat-1 genome (FIG. 11). A Northern analysis was next performed to show that the YZ-15 clone expressed the PLJ-hPTH1-34 transgene, as evidenced by the presence of specific PLJ-hPTH1-34 transcripts (FIG. 12).

EXAMPLE IV

PARATHYROID HORMONE POLYPEPTIDE EXPRESSION AND ACTIVITY

A sensitive and specific radioimmunoassay was performed to demonstrate that the YZ-15 cells expressed and secreted a recombinant hPTH1-34 molecule (Table 2). The radioimmunoassay was performed on media from transduced Rat-1 clones. To quantify secretion of the recombinant hPTH-1-34 peptide produced by YZ-15 cells, the culture medium from one 100 mm confluent dish was collected over a 24 hour period and assayed with the NH2-terminal hPTH RIA kit (Nichols Institute Diagnostics) according to the manufacturer's protocol. PLJ-hPTH1-87 cells and BAG cells served as positive and negative controls, respectively.

Protein concentrations in Table 2 are expressed as the average of three assays plus the standard deviation (in parenthesis). The concentration of the 1-34 and full length (1-84) peptides was determined relative to a standard curve generated with commercially available reagents (Nichols Institute Diagnostics).

Table 2

CELL LINES	PTH (pg/ml)
YZ-15	247 (± 38)
PLJ-hPTH1-84	2616 (± 372)
BAG	13 (± 3)

As shown in Table 2, PTH expression was detected in both YZ-15 cells and PLJ-hPTH1-84 cells. BAG cells produced no detectable PTH and served as a baseline for the RIA. These results demonstrate that YZ-15 cells expressed recombinant hPTH1-34 protein.

The recombinant hPTH1-34 molecule was added to rat

osteosarcoma cells and a cAMP response assay conducted in order to determine whether the secreted molecule had biological activity. Unconcentrated media was collected from YZ-15 cells, PLJ-hPTH1-84 cells, and BAG cells and was used to treat ROS17/2.8 cells for 10 minutes, as described (Majmudar *et al.*, 1991). cAMP was then extracted from treated cells and quantified by RIA (Table 3). The amount of cAMP shown is the average of three assays. The standard deviation of the mean is shown in parenthesis.

Table 3

CELL LINES	cAMP (pmol)
YZ-15	20.3 (± 0.25)
PLJ-hPTH184	88.5 (± 4.50)
BAG	7.6 (± 0.30)

A cAMP response was induced by the recombinant PTH secreted by the YZ-15 cells and by PLJ-hPTH1-84 cells. BAG cells produced no PTH and served as the baseline for the cAMP assay. These results provide direct *in vitro* evidence that the PLJ-hPTH1-34 transgene directs the expression and secretion of a functional osteotropic agent.

EXAMPLE V

<u>BONE MORPHOGENETIC PROTEIN (BMP) GENE CONSTRUCTS</u>

The murine bone morphogenetic protein-4 (BMP-4) was chosen as the next of the osteotropic genes to be incorporated into an expression vector for use in promoting bone repair and regeneration.

A full length murine BMP-4 cDNA was generated by screening a murine 3T3 cell cDNA library (Stratagene). The human sequence for BMP-4 is well known to those of

skill in the art and has been deposited in Genbank. Degenerate oligonucleotide primers were prepared and employed in a standard PCR™ to obtain a murine cDNA sequence.

The ends of the cDNA clone were further modified using the polymerase chain reaction so that the full length cDNA (5'→3' direction) encodes the natural murine initiator Met codon, the full length murine coding sequence, a 9 amino acid tag (known as the HA epitope), and the natural murine stop codon. The amino acid sequence encoded by the murine BMP-4 transgene is shown in FIG. 24; this entire sequence, including the tag, is represented by SEQ ID NO:1.

Placement of the HA epitope at the extreme carboxy terminus should not interfere with the function of the recombinant molecule sequence *in vitro* or *in vivo*. The advantage of the epitope is for utilization in immunohistochemical methods to specifically identify the recombinant murine BMP-4 molecule in osteotomy tissues *in vivo*, *e.g.*, the epitope can be identified using a commercially available monoclonal antibody (Boehringer-Mannheim), as described herein.

Studies to demonstrate that the murine BMP-4 transgene codes for a functional osteotropic agent include, for example, (a) transfection of COS cells and immunoprecipitation of a protein band of the correct size using a monoclonal anti-HA antibody (Boehringer-Mannheim); and (b) a quantitative *in vivo* bone induction bioassay (Sampath and Reddi, 1981) that involves implanting proteins from the medium of transfected COS cells beneath the skin of male rats and scoring for new bone formation in the ectopic site.

EXAMPLE VI

DETECTION OF mRNA BY TISSUE *IN SITU* HYBRIDIZATION

The following technique describes the detection of mRNA in tissue obtained from the site of bone regeneration. This may be useful for detecting expression of the transgene mRNA itself, and also in detecting expression of hormone or growth factor receptors or other molecules. This method may be used in place of, or in addition to, Northern analyses, such as those described in FIG. 13.

DNA from a plasmid containing the gene for which mRNA is to be detected is linearized, extracted, and precipitated with ethanol. Sense and antisense transcripts are generated from 1 mg template with T3 and T7 polymerases, *e.g.*, in the presence of [^{35}S] UTP at >6 mCi/ml (Amersham Corp., >1200 Ci/mmol) and 1.6 U/ml RNasin (Promega), with the remaining *in vitro* transcription reagents provided in a kit (SureSite, Novagen Inc.). After transcription at 37°C for 1 hour, DNA templates are removed by a 15 minute digestion at 37°C with 0.5 U/ml RNase-free DNase I, extracted, and precipitated with ethanol. Riboprobes are hydrolyzed to an average final length of 150 bp by incubating in 40 mM $NaHCO_3$, 60 mM Na_2CO_3, 80 mM DTT at 60°C, according to previously determined formula. Hydrolysis is terminated by addition of sodium acetate, pH 6.0, and glacial acetic acid to 0.09 M and 0.005% (v/v), respectively, and the probes are then ethanol precipitated, dissolved in 0.1 M DTT, counted, and stored at -20°C until use.

RNase precautions are taken in all stages of slide preparation. Bouins fixed, paraffin embedded tissue sections are heated to 65°C for 10 minutes, deparaffinized in 3 changes of xylene for 5 minutes, and rehydrated in a descending ethanol series, ending in

phosphate-buffered saline (PBS). Slides will be soaked in 0.2 N HCl for 5 min., rinsed in PBS, digested with 0.0002% proteinase K in PBS for 30 minutes at 37°C and rinsed briefly with DEPC-treated water. After equilibrating for 3 minutes in 0.1 M triethanolamine-HCl (TEA-HCl), pH 8.0, sections are acetylated in 0.25% (v/v) acetic anhydride in 0.1 M TEA-HCl for 10 minutes at room temperature, rinsed in PBS, and dehydrated in an ascending ethanol series. Each section receives 100-200 ml prehybridization solution (0.5 mg/ml denatured RNase-free tRNA (Boehringer-Mannheim), 10 mM DTT, 5 mg/ml denatured, sulfurylated salmon sperm DNA, 50% formamide, 10% dextran sulfate, 300 mM NaCl, 1X RNase-free Denhardt's solution (made with RNase-free bovine serum albumin, Sigma), 10 mM Tris-HCl, pH 7.4, 1 mM EDTA) and then incubate on a 50°C slide warmer in a humidified enclosure for 2 hours. The sulfurylated salmon-sperm DNA blocking reagent is used in both prehybridization and hybridization solutions to help reduce nonspecific binding to tissue by ^{35}SH groups on the probe. It is prepared by labeling RNase-free salmon sperm DNA (Sigma) with non-radioactive α-thio-dCTP and α-thio-dATP (Amersham) in a standard random oligonucleotide-primed DNA labeling reaction. Excess prehybridization solution is removed with a brief rinse in 4X SSC before application of probe.

Riboprobes, fresh tRNA and sulfurylated salmon sperm DNA will be denatured for 10 minutes at 70°C, and chilled on ice. Hybridization solution, identical to prehybridization solution except with denatured probe added to 5×10^6 CPM/ml, is applied and slides incubated at 50°C overnight in sealed humidified chambers on a slide warmer. Sense and antisense probes are applied to serial sections. Slides are rinsed 3 times in 4X SSC, washed with 2X SSC, 1 mM DTT for 30 min. at 50°C, digested with RNase A (20 mg/ml RNase A, 0.5 M NaCl, 10

mM Tris, pH 8.0, 1 mM EDTA, pH 8.0) for 30 min. at 37°C, and rinsed briefly with 2X SSC, 1 mM DTT. Three additional washes are performed, each at 50°C for 30 minutes: once in 2X SSC, 50% formamide, 1 mM DTT, and twice in 1X SSC, 0.13% (w/v) sodium pyrophosphate, 1 mM DTT.

Slides are dehydrated in an ascending ethanol series (with supplementation of the dilute ethanols (50% and 70%) with SSC and DTT to 0.1X and 1 mM, respectively). Slides are exposed to X-ray film for 20-60 hours to visualize overall hybridization patterns, dipped in autoradiographic emulsion (Kodak NTB-2, diluted to 50% with 0.3 M ammonium acetate), slowly dried for 2 hours, and exposed (4°C) for periods ranging from 8 days to 8 weeks. After developing emulsion, sections are counter strained with hematoxylin and eosin, dehydrated, and mounted with xylene-based medium. The hybridization signal is visualized under darkfield microscopy.

The above *in situ* hybridization protocol may be used, for example, in detecting the temporal and spatial pattern of PTH/PTHrP receptor expression. A suitable rat PTH/PTHrP receptor cDNA probe (R15B) is one that consists of a 1810 bp region encoding the full length rat bone PTH/PTHrP receptor (Abou-Samra *et al.*, 1992). The cDNA fragment is subcloned into pcDNA 1 (Invitrogen Corp., San Diego, CA) and is cut out using *Xba*I and *Bam*HI. This probe has provided positive signals for northern blot analysis of rat, murine, and human osteoblastic cell lines, rat primary calvarial cells, and murine bone tissue. The pcDNA I plasmid contains a T7 and SP6 promoter that facilitate the generation of cRNA probes for *in situ* hybridization. The full length transcript has been used to detect PTH/PTHrP receptor in sections of bone (Lee *et al.*, 1994). The PTHrP cDNA probe (Yasuda *et al.*, 1989) is a 400 bp subcloned fragment in

pBluescript (Stratagene). This probe has been used for *in situ* hybridization, generating an antisense cRNA probe using *Bam*HI cleavage and the T3 primer and a sense cRNA probe using *Eco*RI cleavage and the T7 primer.

EXAMPLE VII

IN VIVO PROTEIN DETECTION FOLLOWING TRANSGENE EXPRESSION

1. **β-galactosidase Transgene**

Bacterial β-galactosidase can be detected immunohistochemically. Osteotomy tissue specimens are fixed in Bouins fixative, demineralized, and then split in half along the longitudinal plane. One-half of each specimen is embedded in paraffin for subsequent immunohistochemical identification of the bacterial β-galactosidase protein.

For immunohistochemistry, cross-Sections (2-3 mm thick) were transferred to poly-L-Lysine coated microscope slides and fixed in acetone at 0°C for at least 20 min. Sections were rehydrated in PBS. Endogenous peroxidase activity was quenched by immersion of tissue sections in 0.1% hydrogen peroxide (in 95% methanol) at room temperature for 10 min, and quenched sections were washed 3x in PBS. In some cases, sectioned calvariae were demineralized by immersion in 4% EDTA, 5% polyvinyl pyrrolidone, and 7% sucrose, pH 7.4, for 24 h at 4°C. Demineralized sections were washed 3x before application for antibodies. Primary antibodies were used without dilution in the form of hybridoma supernatant. Purified antibodies were applied to tissue sections at a concentration of 5 mg/ml. Primary antibodies were detected with biotinylated rabbit antimouse IgG and peroxidase conjugated streptavidin (Zymed Histostain-SPkit). After peroxidase staining, sections were counterstained with hematoxylin.

Bacterial β-gal can also be detected by substrate utilization assays. This is conducted using commercially available kits (*e.g.*, Promega) according to the manufacturers' instructions.

2. Luciferase Transgene

Luciferase can be detected by substrate utilization assays. This is conducted using commercially available kits (*e.g.*, Promega) according to the manufacturers' instructions.

3. PTH Transgenes

Recombinant PTH, such as hPTH1-34 peptide, is assayed in homogenates of osteotomy gap tissue, for example, using two commercially available radioimmunoassay kits according to the manufacturer's protocols (Nichols Institute Diagnostics, San Juan Capistrano, CA).

One kit is the Intact PTH-Parathyroid Hormone 100T Kit. This radioimmunoassay utilizes an antibody to the carboxy terminus of the intact hormone, and thus is used to measure endogenous levels of hormone in gap osteotomy tissue. This assay may be used to establish a baseline value PTH expression in the rat osteotomy model.

The second kit is a two-site immunoradiometric kit for the measurement of rat PTH. This kit uses affinity purified antibodies specific for the amino terminus of the intact rat hormone (PTH1-34) and thus will measure endogenous PTH production as well as the recombinant protein. Previous studies have shown that these antibodies cross-react with human PTH and thus are able to recognize recombinant molecules *in vivo*.

Values obtained with kit #1 (antibody to the carboxy terminus) are subtracted from values obtained with kit #2 (antibody to the amino terminus) to obtain an accurate and sensitive measurements. The level of recombinant peptide is thus correlated with the degree of new bone formation.

4. BMP Transgene

Preferably, BMP proteins, such as the murine BMP-4 transgene peptide product, are detected immunohistochemically using a specific antibody that recognizes the HA epitope (Majmudar *et al.*, 1991), such as the monoclonal antibody available from Boehringer-Mannheim. Antibodies to BMP proteins themselves may also be used. Such antibodies, along with various immunoassay methods, are described in U.S. Patent 4,857,456, incorporated herein by reference.

Osteotomy tissue specimens are fixed in Bouins fixative, demineralized, and then split in half along the longitudinal plane. One-half of each specimen is embedded in paraffin for subsequent immunohistochemical identification of the recombinant murine BMP-4 molecule.

EXAMPLE VIII

DIRECT GENE TRANSFER INTO REGENERATING BONE *IN VIVO*

To assess the feasibility of direct gene transfer into regenerating bone *in vivo*, marker gene transfer into cells in the rat osteotomy model was employed. These studies involved two marker genes: bacterial β-galactosidase and insect luciferase.

Aliquots of a fibrous collagen implant material were soaked in solutions of pure marker gene DNA. The implant

materials were then placed in the osteotomy site, and their expression determined as described above.

It was found that both marker genes were successfully transferred and expressed, without <u>any</u> failures, as demonstrated by substrate utilization assays (FIG. 5A, FIG. 5B, FIG. 6A, FIG. 6B, FIG. 6C and FIG. 6D). Since mammalian cells do not normally synthesize either marker gene product, this provides direct evidence that osteotomy repair cells were transfected *in vivo* and then expressed the β-galactosidase and luciferase transgenes as a functional enzymes.

EXAMPLE IX

ADENOVIRAL GENE TRANSFER INTO REGENERATING BONE *IN VIVO*

One of the alternative methods to achieve *in vivo* gene transfer into regenerating bone is to utilize an adenovirus-mediated transfer event. Successful adenoviral gene transfer of a marker gene construct into bone repair cells in the rat osteotomy model has been achieved (FIG. 23A, FIG. 23B, and FIG. 23C).

The inventors employed the adenoviral vector pAd. CMV*lacZ*, which is an example of a replication-defective adenoviral vector which can replicate in permissive cells (Stratford-Perricaudet *et al.*, 1992). In pAd.CMV*lacZ*, the early enhancer/promoter of the cytomegalovirus (CMV) is used to drive transcription of *lacZ* with an SV40 polyadenylation sequence cloned downstream from this reporter (Davidson *et al.*, 1993).

The vector pAd.RSV4 is also utilized by the inventors. This vector essentially has the same backbone as pAdCMV*lacZ*, however the CMV promoter and the single *Bgl*II cloning site have been replaced in a cassette-like fashion with *Bgl*II fragment that consists of an RSV

promoter, a multiple cloning site, and a poly(A^+) site. The greater flexibility of this vector is contemplated to be useful in subcloning osteotropic genes, such as the hPTH1-34 cDNA fragment, for use in further studies.

To generate recombinant PTH adenovirus, a 100-mm dish of 293 cells is transfected using calcium phosphate with 20 mg of a plasmid construct, *e.g.*, the plasmid containing the hPTH1-34 insert linearized with *Nhe*I, plus 2 mg of wild type adenovirus DNA digested with *Xba*I and *Cla*I. The adenovirus DNA is derived from adenovirus type 5, which contains only a single *Xba*I and *Cla*I sites and has a partial deletion of the E3 region. Approximately 7 days post-transfection, cells and media are harvested and a lysate prepared by repeated freeze-thaw cycles. This lysate is diluted and used to infect 60 -mm dishes of confluent 293 cells for 1 hour. The cells are then overlaid with 0.8% agar/1X MEM/2% calf serum/12.5 mM $MgCl_2$. Ten days post-infection, individual plaques are to be picked and used to infect 60-mm dishes of 293 cells to expand the amount of virus. Positive plaques are selected for further purification and the generation of adenoviral stocks.

To purify recombinant adenovirus, 150-mm dishes of 75-90% confluent 293 cells are infected with 2-5 PFU/cell, a titer that avoids the potential cytotoxic effects of adenovirus. Thirty hours post-infection, the cells are rinsed, removed from the dishes, pelleted, and resuspended in 10 mM Tris-HCl, pH 8.1. A viral lysate is generated by three freeze-thaw cycles, cell debris is removed by centrifugation for 10 min. at 2,000 rpm, and the adenovirus is purified by density gradient centrifugation. The adenovirus band is stored at -20°C in sterile glycerol/BSA until needed.

The solution of virus particles was sterilized and

incubated with the implant material (from 6 min to overnight), and the virus-impregnated material was implanted into the osteotomy gap; where viral infection of cells clearly occurred. The results obtained clearly demonstrated the exquisite specificity of the anti-β-gal antibody (Sambrook *et al.*, 1989), and conclusively demonstrated expression of the marker gene product in chondrocyte-like cells of the osteotomy gap. The nuclear-targeted signal has also been observed in pre-osteoblasts.

EXAMPLE X

TRANSFER OF AN OSTEOTROPIC GENE STIMULATES BONE REGENERATION/REPAIR *IN VIVO*

In order for a parathyroid hormone (PTH) transgene to function as an osteotropic agent, it is likely that there is a requirement for the PTH/PTHrP receptor to be expressed in the bone repair tissue itself. Therefore, the inventors investigated PTH/PTHrP receptor expression in the rat osteotomy model.

A Northern analysis of poly-A(+) RNA was conducted which demonstrated that the PTH/PHTrP receptor was expression in osteotomy repair tissue (FIG. 13).

The inventors next investigated whether gene transfer could be employed to create transfected cells that constitutively express recombinant hPTH1-34 *in vivo*, and whether this transgene can stimulate bone formation. The rate of new bone formation is analyzed as follows. At necropsy the osteotomy site is carefully dissected for histomorphometric analysis. The A-P and M-L dimensions of the callus tissue are measured using calipers. Specimens are then immersion fixed in Bouins fixative, washed in ethanol, and demineralized in buffered formic acid. Plastic embedding of decalcified materials is used because of the superior dimensional stability of

methacrylate during sample preparation and sectioning.

Tissue blocks are dehydrated in increasing alcohol concentrations and embedded. 5 mm thick sections are cut in the coronal plane using a Reichert Polycut microtome. Sections are prepared from midway through the width of the marrow cavity to guard against a sampling bias. Sections for light microscopy are stained using a modified Goldner's trichrome stain, to differentiate bone, osteoid, cartilage, and fibrous tissue. Sections are cover-slipped using Eukitt's mounting medium (Calibrated Instruments, Ardsley, NY). Histomorphometric analyses are performed under brightfield using a Nikon Optiphot Research microscope. Standard point count stereology techniques using a 10 mm x 10 mm eyepiece grid reticular are used.

Total callus area is measured at 125X magnification as an index of the overall intensity of the healing reaction. Area fractions of bone, cartilage, and fibrous tissue are measured at 250 X magnification to examine the relative contribution of each tissue to callus formation. Since the dimensions of the osteotomy gap reflect the baseline (time 0), a measurement of bone area at subsequent time intervals is used to indicate the rate of bone infill. Statistical significance is assessed using analysis of variance, with post-hoc comparisons between groups conducted using Tukey's studentized range t test.

In the 5-mm rat osteotomy model described above, it was found that PTH transgene expression can stimulate bone regeneration/repair in live animals (FIG. 6A, FIG. 6B, FIG. 6C, and FIG. 6D). This is a particularly important finding as it is known that hPTH1-34 is a more powerful anabolic agent when given intermittently as opposed to continuously, and it is the continuous-type delivery that results from the gene transfer methods used

here.

Although the present inventors have already demonstrated success of direct gene transfer into regenerating bone *in vivo*, the use of *ex vivo* treatment protocols is also contemplated. In such embodiments, bone progenitor cells would be isolated from a particular animal or human subject and maintained in an *in vitro* environment. Suitable areas of the body from which to obtain bone progenitor cells are areas such as the bone tissue and fluid surrounding a fracture or other skeletal defect (whether or not this is an artificially created site) and from the bone marrow. Isolated cells would then be contacted with the DNA (or recombinant viral) composition, with, or preferably without, a matrix, when the cells would take up the DNA (or be infected by the recombinant virus). The stimulated cells would then be returned to the site in the animal or patient where bone repair is to be stimulated.

EXAMPLE XI

TRANSFER OF GENES TO ACHILLES' TENDON AND TO CRUCIATE LIGAMENT *IN VIVO*

The studies on regenerating bone described above complement others by the inventors in which gene transfer was successfully employed to introduce genes into Achilles' tendon (FIG 3A, FIG. 3B, FIG. 3C, FIG. 3D, and FIG. 3E) and cruciate ligament (FIG. 4).

The Achilles' tendon consist of cells and extracellular matrix organized in a characteristic tissue architecture. Tissue wounding can disrupt this architecture and stimulate a wound healing response. The wounded tendon will regenerate, as opposed to scar, if its connective tissue elements remain approximately intact. Regeneration is advantageous because scar tissue is not optimally designed to support normal mechanical

function. Segmental defects in tendon due to traumatic injury may be treated with biological or synthetic implants that encourage neo-tendon formation. This strategy is limited, however, by the availability of effective (autologous) biological grafts, the long term stability and compatibility of synthetic prostheses, and the slow rate of incorporation often observed with both types of implants.

The inventors hypothesized that the effectiveness of biological grafts may be enhanced by the over-expression of molecules that regulate the tissue regeneration response. Toward this end, they developed a model system in which segmental defects in Achilles' tendon are created and a novel biomaterial, is used as a tendon implant/molecular delivery agent. In the present example, the ability to deliver and express marker gene constructs into regenerating tendon tissue is demonstrated.

Plasmid (pSVβgal, Promega) stock solutions were prepared according to standard protocols (Sambrook *et al.*, 1989). SIS graft material was prepared from a segment of jejunum of adult pigs (Badylak *et al.*, 1989). At harvest, mesenteric tissues were removed, the segment was inverted, and the mucosa and superficial submucosa were removed by a mechanical abrasion technique. After returning the segment to its original orientation, the serosa and muscle layers were rinsed, sterilized by treatment with dilute peracetic acid, and stored at 4°C until use.

Mongrel dogs (all studies) were anesthetized, intubated, placed in right-lateral recumbency upon a heating pad, and maintained with inhalant anesthesia. A lateral incision from the musculotendinous junction to the plantar fascia was used to expose the Achilles'

tendon. A double thickness sheet of SIS was wrapped around a central portion of the tendon, both ends were sutured, a 1.5 cm segment of the tendon was removed through a lateral opening in the graft material, and the graft and surgical site were closed. The leg was immobilized for 6 weeks and then used freely for 6 weeks. Graft tissues were harvested at time points indicated below, fixed in Bouins solution, and embedded in paraffin. Tissue sections (8 μm) were cut and used for immunohistochemistry.

In an initial study, SIS material alone (SIS-alone graft) engrafted and promoted the regeneration of Achilles' tendon following the creation of a segmental defect in mongrel dogs as long as 6 months post surgery. The remodeling process involved the rapid formation of granulation tissue and eventual degradation of the graft. Scar tissue did not form, and evidence of immune-mediated rejection was not observed.

In a second study, SIS was soaked in a plasmid DNA solution (SIS+plasmid graft) and subsequently implanted as an Achilles' tendon graft (n=2 dogs) or a cruciate ligament graft (n=2 dogs) in normal mongrel dogs. A pSVβgal plasmid that employs simian virus 40 regulatory sequences to drive β-galactosidase (β-gal) activity was detectable by immunohistochemistry using a specific antibody in 4/4 animals. As a negative control, β-gal activity was not detected in the unoperated Achilles' tendon and cruciate ligament of these animals. It appeared, therefore, that SIS facilitated the uptake and subsequent expression of plasmid DNA by wound healing cells in both tendon and ligament.

A third study was designed to evaluate the time course of β-gal transgene expression. SIS + plasmid grafts were implanted for 3, 6, 9, and 12 weeks (n=2 dogs

pr time point) and transgene expression was assayed by immunohistochemistry and by *in situ* hybridization. Cross-sections (8-μm) of Bouins fixed, paraffin embedded tissue were cut and mounted on ProbeOn Plus slides (Fisher). Immunohistochemistry was performed according to the protocol provided with the Histostain-SP kit (Zymed). In brief, slides were incubated with a well characterized anti-β-galactosidase antibody (1:200 dilution, 5'→3'), washed in PBS, incubated with a biotinylated second antibody, washed, stained with the enzyme conjugate plus a substrate-chromogen mixture, and then counterstained with hematoxylin and eosin.

Bacterial β-gal activity was detected in tendons that received the SIS+plasmid graft (8/8 animals). Although not rigorously quantitative, transgene expression appeared to peak at 9-12 weeks. Bacterial β-gal gene expression was not detected in animals that received SIS-alone grafts (N=2, 3 weeks and 12 weeks). Again, scar tissue did not form and evidence of immune-mediated rejection was not observed.

This study demonstrated that the mucosal biomaterial SIS can function as an autologous graft that promotes the regeneration of tissues such as Achilles' tendon and anterior cruciate ligament. SIS can also be used to deliver a marker gene construct to regenerating tissue.

EXAMPLE XIII
MECHANICAL PROPERTIES OF NEW BONE FORMATION

The mechanical properties of new bone formed during gene transfer may be measured using, *e.g.*, whole bone torsion tests which create a stress state in which the maximum tensile stresses will occur on planes that lie obliquely to the bone's longitudinal axis. Such tests may provide important inferences about the mechanical

anisotropy of callus tissue and the degree of osseous integration of new bone tissue. These tests are particularly advantageous in the evaluation of fracture specimens, *e.g.*, the irregular shape of callus tissue typically precludes the use of whole bone 4-point bending tests because it is impossible to reproducibly align the points from specimen to specimen.

Femurs are tested on an MTS Servohydraulic Testing Machine while moist and at room temperature. A torque sensor and rotary variable displacement transduces provides data for torque-angular displacement curves. Specially designed fixtures support each bone near the metaphyseal-diaphyseal junctions, and apply a 2-point load to the diaphysis. Tests are conducted at a constant rate of displacement equal to 20 degrees/sec. A 250 inch-ounce load cell measures the total applied force. All bones are tested while moist and room temperature. Torque and angular displacement data are acquired using an analog-to-digital converter and a Macintosh computer and software. From this data, the following variables are calculated: a) maximum torque, b) torsional stiffness, the slope of the pre-yield portion of the curve determined from a linear regression of the data, c) energy to failure, the area under the torque-angular displacement curve to the point of failure, and d) the angular displacement ratio, the ratio of displacement at failure to displacement at yield. Statistical significance is determined Analysis of Variance followed by multiple comparisons with appropriate corrections (*e.g.*, Bonferroni).

This invention also provides a means of using osteotropic gene transfer in connection with reconstructive surgery and various bone remodelling procedures. The techniques described herein may thus be employed in connection with the technology described by

Yasko *et al.*, 1992; Chen *et al.*, 1991; and Beck *et al.*, 1991, each incorporated herein by reference.

EXAMPLE XIV

TYPE II COLLAGEN PROMOTES NEW BONE GROWTH

Certain matrix materials are capable of stimulating at least some new growth in their own right, *i.e.*, are "osteoconductive materials". Potential examples of such materials are well known in the field of orthopedic research and include preparations of hydroxyapatite; preparations of crushed bone and mineralized collagen; PLGA block copolymers and polyanhydride. The ability of these materials to stimulate new bone formation distinguishes them from inert implant materials such as methylcellulose, which have in the past been used to deliver BMPs to sites of fracture repair.

This Example relates to a study using the rat osteotomy model with implants made of collagen type I (Sigma), collagen type II (Sigma), and UltraFiber™ (Norian Corp.). These materials have been placed *in situ* without DNA of any type. Five animals received an osteotomy with 10 mg of a type II collagen implant alone (10 mg refers to the original quantity of lyophilized collagen). Five of five control animals received an osteotomy with 10 mg of a type I collagen implant alone. Animals were housed for three weeks after surgery and then sacrificed.

The results of these studies were that SIS appeared to retard new bone formation; type I collagen incited a moderately intense inflammatory response; and UltraFiber™ acted as an osteoconductive agent. The type II collagen implant studies yielded surprising results in that 10 mg of this collagen was found to promote new bone formation in the 5-mm osteotomy model (FIG. 22A, FIG. 22B, and FIG.

22C). New bone - bridging the osteotomy gap - was identified three weeks after surgery in 5/5 animals that received a type II collagen implant alone (*i.e.*, minus DNA of any type). In contrast, fibrous granulation tissue, but no evidence of new bone formation, was obtained in 5/5 animals receiving a type I collagen implant alone.

Radiographic analysis demonstrated conclusively that all animals receiving an osteotomy with a type II collagen implant without exception showed radio-dense material in the osteotomy gap (FIG. 22A). In sharp contrast, radiographic analysis of all animals receiving a type I collagen implant revealed no radio-dense material forming in the osteotomy gap (FIG. 22B). The arrow in FIG. 22A point to the new bone growth formed in the osteotomy gap of type II collagen implanted-animals. No such new bone growth was observed in the animals receiving type I collagen implants (FIG. 22B).

FIG. 22C demonstrates the results of the osteotomy with a type II collagen implant. The arrow points to the area of new bone formed in the osteotomy gap. In contrast, only fibrous granulation tissue was identified in the type I collagen gap.

Previous studies have suggested that type II collagen plays only a structural role in the extracellular matrix. The results of the type II collagen implant studies are interesting because they demonstrate a novel and osteoconductive role for type II collagen during endochondral bone repair. To further optimize the osteoconductive potential of type II collagen, a yeast expression vector that encodes for type II collagen (full length $\alpha 1(II)$ collagen) will be employed to produce recombinant $\alpha 1(II)$ collagen protein.

EXAMPLE XV

IDENTIFICATION OF FURTHER OSTEOTROPIC GENES:

ISOLATION OF A NOVEL LATENT TGF-β BINDING PROTEIN-LIKE (LTBP-3) GENE

The TGF-βs represent a family of structurally related molecules with diverse effects on mammalian cell shape, growth, and differentiation (Roberts and Sporn, 1990). Initially synthesized as a precursor consisting of an amino-terminal propeptide followed by mature TGF-β, two chains of nascent pro-TGF-β associate in most tissues to form a Mr ~106,000 inactive disulfide-bonded dimer. Homodimers are most common, but heterodimers have also been described (Cheifetz *et al.*, 1987; Ogawa *et al.*, 1992). During biosynthesis the mature TGF-β dimer is cleaved from the propeptide dimer. TGF-β latency results in part from the non-covalent association of propeptide and mature TGF-β dimers (Pircher *et al.*, 1984 and 1986; Wakefield *et al.*, 1987; Millan *et al.*, 1992; see also Miyazono and Heldin, 1989). Consequently, the propeptide dimer is often referred to as the latency associated protein (LAP), and LAP plus the disulfide-bonded TGF-β dimer are also known as the small latent complex. In the extracellular space small latent complexes must be dissociated to activate mature TGF-β. The mechanism of activation of the latent complex is thought to be one of the most important steps governing TGF-β effects (Lyons *et al.*, 1988; Antonelli-Orlidge *et al.*, 1989; Twardzik *et al.*, 1990; Sato *et al.*, 1993).

In certain lines of cultured cells small latent growth factor complexes may contain additional high molecular weight proteins. The best characterized of these high molecular weight proteins is the latent TGF-β binding protein, or LTBP (Miyazono *et al.*, 1988; Kanzaki *et al.*, 1990; Tsuji *et al.*, 1990; Olofsson *et al.*, 1992; Taketazu *et al.*, 1994). LTBP produced by different cell

types is heterogeneous in size, perhaps because of alternative splicing or because of tissue-specific proteolytic processing (Miyazono *et al.*, 1988; Wakefield *et al.*, 1988; Kanzaki *et al.*, 1990; Tsuji *et al.*, 1990). Latent TGF-β complexes that contain LTBP are known as large latent complexes. LTBP has no known covalent linkage to mature TGF-β, but rather it is linked by a disulfide bond to LAP.

Two LTBPs have been isolated to date. The deduced human LTBP-1 amino acid sequence is comprised of a signal peptide, 16 epidermal growth factor-like repeats with the potential to bind calcium (EGF-CB repeats), 2 copies of a unique motif containing 8 cysteine residues, an RGD cell attachment motif, and an 8 amino acid motif identical to the cell binding domain of the laminin B2 chain (Kanzaki *et al.*, 1990). There is evidence that LTBP-1 binds calcium, which, in turn, induces a structural change that protects LTBP from proteolytic attack (Colosetti *et al.*, 1993). LTBP-2 shows 41% sequence identity to LTBP-1, and its structural domains show a similar overall organization (Moren *et al.*, 1994).

While the functions of LTBP-1 and LTBP-2 presently are unknown, several ideas have been put forward in the literature. First, LTBP may regulate the intracellular biosynthesis of latent TGF-β precursors. Cultured erythroleukemia cells efficiently assemble and secrete large latent TGF-β complexes, whereas they slowly secrete small latent TGF-β complexes that contain anomalous disulfide bonds (Miyazono *et al.*, 1991; Miyazono *et al.*, 1992). Therefore, LTBP may facilitate the normal assembly and secretion of latent TGF-β complexes. Second, LTBP may target latent TGF-β to specific types of connective tissue. Recent evidence suggests that the large latent TGF-β complex is covalently bound to the extracellular matrix via LTBP (Taipale *et al.*, 1994).

Based on these observations, LTBP has been referred to as a "matrix receptor", *i.e.* a secreted protein that targets and stores latent growth factors such as TGF-β to the extracellular matrix. Third, LTBP may modulate the activation of latent complexes. This idea is based in part on recent evidence which suggests that mature TGF-β is released from extracellular storage sites by proteases such as plasmin and thrombin and that LTBP may protect small latent complexes from proteolytic attack (Falcone *et al.*, 1993; Benezra *et al.*, 1993; Taipale *et al.*, 1994), *i.e.* protease activity may govern the effect of TGF-β in tissues, but LTBP may modulate this activity. Fourth, LTBP may plays an important role in targeting the latent TGF-β complex to the cell surface, allowing latent TGF-β to be efficiently activated (Flaumenhaft *et al.*, 1993).

A. MATERIALS AND METHODS

1. cDNA Cloning

Aliquots (typically 40-50,000 PFU) of phage particles from a cDNA library in the λZAPII® vector made from NIH 3T3 cell mRNA (Stratagene) and fresh overnight XL1-Blue™ cells (grown in Luria broth supplemented with 0.4% maltose in 10 mM $MgSO_4$) were mixed, incubated for 15 min. at 37°C, mixed again with 9 ml of liquid (50°C) top layer agarose (NZY broth plus 0.75% agarose), and then spread evenly onto freshly poured 150 mm NZY-agar plates. Standard methods were used for the preparation of plaque-lifts and filter hybridization (42°C, in buffer containing 50% formamide, 5X SSPE, 1X Denhardt's, 0.1% SDS, 100 mg/ml salmon sperm DNA, 100 mg/ml heparin). Filters were washed progressively to high stringency (0.1X SSC/0.1% SDS, 65°C). cDNA probes were radiolabeled by the nick translation method using commercially available reagents and protocols (Nick Translation Kit,

Boehringer Mannheim). Purified phage clones were converted to pBluescript® plasmid clones, which were sequenced using Sequenase (v2.0) as described (Chen *et al.*, 1993; Yin *et al.*, 1995). Sequence alignment and identity was determined using sequence analysis programs from the Genetics Computer Group (MacVector).

2. Tissue *In Situ* Hybridization

To prepare normal sense and antisense probes, a unique 342 bp fragment from the 3' untranslated region (+3973 to +4314, counting the "A" of the initiator Met codon as +1; see "ish", Fig. 1) was subcloned into the pBSKS+ plasmid (Stratagene, Inc.). Template DNA was linearized with either *Eco*RI or *Bam*HI, extracted, and precipitated with ethanol. Sense and antisense transcripts were generated from 1 mg template with T3 and T7 polymerases in the presence of [^{35}S]UTP at >6 mCi/ml (Amersham, >1200 Ci/mmol) and 1.6 U/ml RNasin (Promega), with the remaining *in vitro* transcription reagents provided in a kit (SureSite, Novagen Inc.). After transcription at 37°C for 1 h, DNA templates were removed by a 15 min. digest at 37°C with 0.5 U/ml RNase-free DNase I, extracted, and precipitated with ethanol. Riboprobes were hydrolyzed to an average final length of 150 bp by incubating in 40 mM $NaHCO_3$, 60 mM Na_2CO_3, 80 mM DTT for ~40 min. at 60°C. Hydrolysis was terminated by addition of sodium acetate, pH 6.0, and glacial acetic acid to .09 M and 0.56% (v/v), respectively, and the probes were then ethanol precipitated, dissolved in 0.1 M DTT, counted, and stored at -20°C until use. Day 8.5-9.0, day 13.5, and day 16.5 mouse embryo tissue sections (Novagen) and the *in situ* hybridization protocol

were exactly as described (Chen *et al.*, 1993; Yin *et al.*, 1995).

3. Northern Analysis

MC3T3-E1 cell poly(A+) RNA (2-10 mg aliquots) was electrophoresed on a 1.25% agarose/2.2 M formaldehyde gel and then transferred to a nylon membrane (Hybond-N, Amersham). The RNA was cross-linked to the membrane by exposure to a UV light source (1.2×10^6 mJ/cm^2, UV Stratalinker 2400, Stratagene) and then pre-hybridized for >15 min. at 65°C in Rapid-Hyb buffer (Amersham, Inc.). A specific cDNA probe consisting solely of untranslated sequence from the 3' end of the transcript was ^{32}P-labeled by random priming and used for hybridization (2 h at 65°C). Blots were washed progressively to high stringency (0.1X SSC/0.1% SDS, 65°C), and then placed against x-ray film with intensifying screens (XAR, Kodak) at -86°C.

4. Antibody Preparation

LTBP-3 antibodies were raised against a unique peptide sequence found in domain #2 (amino acids 155-167). Peptide #274 (GESVASKHAIYAVC) (SEQ ID NO:16) was synthesized using an ABI model 431A synthesizer employing FastMoc chemistry. The sequence was confirmed using an ABI473 protein sequencer. A cysteine residue was added to the carboxy-terminus to facilitate crosslinking to carrier proteins. For antibody production, the synthetic peptide was coupled to rabbit serum albumin (RSA) using MBS (*m*-maleimidobenzoic acid-N-hydroxysuccinimide ester) at a substitution of 7.5 mg peptide per mg of RSA. One mg of the peptide-RSA conjugate in 1 ml of Freund's complete adjuvant was injected subcutaneously at 10 different sites along the backs of rabbits. Beginning at 3 weeks after initial

immunization, the rabbits were given bi-weekly booster injections of 1 mg peptide-RSA in 100 ul of Freund's incomplete adjuvant. IgG was prepared by mixing immune serum with caprylic acid (0.7 ml caprylic acid per ml serum), stirring for 30 min., and centrifuging at 5,000 x *g* for 10 min. The supernatant was decanted and dialyzed against two changes of phosphate buffered saline (PBS) overnight at 4°C. The antibody solution was then affinity purified by passing it over a column containing the immunizing peptide coupled to Affi-gel 10 affinity support. Bound antibodies were eluted with 0.2 M glycine (pH 2.3), immediately dialyzed against PBS, and concentrated to 1 mg/ml. prior to storage at -70°C.

5. Transfection

Transient transfection was performed using standard protocols (Sambrook *et al*., 1989). Briefly, subconfluent cells (covering ~20% of a 100 mm plastic tissue culture dish) were washed 2x in DMEM tissue culture medium (GIBCO) and then incubated for 3 hrs. at 37°C in a sterile mixture of DEAE-dextran (0.25 mg/ml), chloroquine (55 mg/ml), and 15 mg plasmid DNA (Courey and Tjian, 1988). Cells then were shocked by incubation with 10% DMSO in sterile PBS for 2 min. at 37°C, washed 2x with DMEM (Sambrook *et al*., 1989), and incubated in DMEM plus 10% fetal calf serum and antibiotics for 72 hr. at 37°C.

6. Immunoprecipitation

For immunoprecipitation, 1 ml of antibody (1:400 final concentration, in PBS-TDS buffer: 0.38 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1% Triton X-100, 0.5% Deoxycholic acid, and 0.1% SDS) was added to 1 ml of radiolabeled medium proteins. The mixture was incubated with shaking at 4°C for 1 hr., protein A-sepharose CL-4B beads were added (200 ml, 10% suspension), and this

mixture was incubated with shaking for one additional hour at 4°C. Immunoprecipitated proteins were pelleted by brief centrifugation, the pellet was washed 6x with PBS-TDS buffer, 2x protein loading dye was added, and the samples were boiled for 5 min. and then fractionated on 4-18% gradient SDS-PAGE (Bonadio *et al.*, 1985). Cold molecular weight markers (200 kDa-14.3 kDa, Rainbow mix, Amersham) were used to estimate molecular weight. The gel was dried and exposed to film for the indicated time at room temperature.

7. Western Analysis

Fractionated proteins within SDS-polyacrylamide gels were transferred to a nitrocellulose filter for 2 hours using Tris-glycine-methanol buffer, pH 8.3 at 0.5 mA/cm². The filter was blocked, incubated with nonfat milk plus antibody (1:1000 dilution) for 2 hr, and washed. Antibody staining was visualized using the ECL Western blotting reagent (Amersham) according to the manufacturer's protocols.

B. RESULTS

In this study, the inventors isolated and characterized a novel murine fibrillin-like cDNA encoding LTBP-3. To clone the murine LTBP-3 gene, cDNA from a 3T3 cell cDNA library was amplified using human fibrillin-1 PCR™ primers under low stringency conditions (*i.e.*, annealing at 37°C initially for 10 cycles, followed by annealing at 60°C for 30 cycles). The results indicated that a murine DNA fragment of unexpectedly low homology (~50%) to human fibrillin-1 was obtained. Molecular cloning of the authentic murine fibrillin-1 transcript was also performed, confirming the human and murine fibrillin-1 coding sequences share >95% sequence identity. The murine fibrillin-1 and PCR™ sequences were

different, which suggested that the PCR™ product may have been derived from a related, fibrillin-like cDNA. The 3T3 cell cDNA library was screened at high stringency using the murine PCR™ product as the probe in order to test this hypothesis. A cDNA walking strategy eventually yielded seven overlapping cDNA clones (FIG. 14). It provides a unique mRNA of 4,314 nucleotides, with an open reading frame of 3,753 nucleotides (SEQ ID NO:2). The deduced molecule is a unique polypeptide of 1,251 amino acids (SEQ ID NO:3). Excluding the signal peptide (21 amino acids), the novel fibrillin-like molecule consists of five structurally distinct regions (Region 1- Region 5), and although similar to murine fibrillin-1 (FIG. 15A), its domain structure is unique as is evidenced by the schematic representation of LTBP-3 shown in FIG. 15B.

Domain #1 is a 28 amino acid segment with a net basic charge (est. pI, 12.36) that may allow for binding acidic molecules in the extracellular matrix (*e.g.*, acidic proteoglycans). Sequences rich in basic amino acids may also function as endoproteolytic processing signals (Barr, 1991; Steiner *et al.*, 1992), which suggests that the NH_2-terminus may be proteolytically processed. Domain #2 extends for of 390 amino acids, consisting of an EGF-like repeat, a 135 amino acid segment that was proline-rich (20.7%) and glycine-rich (11.8%) but not cysteine-rich, a Fibmotif (Pereira *et al.*, 1993), an EGF-CB repeat, and a TGF-bp repeat. Domain #3 is a 113 amino acid segment characterized by its high proline content (21%). Domain #4 extends for 678 amino acids and consists of 14 consecutive cysteine-rich repeats. Based on structural homologies, 12/14 repeats were epidermal growth factor-calcium binding (EGF-CB) motifs (Handford *et al.*, 1991), whereas 2/14 were transforming growth factor-β-binding protein (TGF-bp) motifs (Kanzaki *et al.*, 1990). Finally, domain #5 is a 22 amino acid segment at the carboxy-terminus. The

conceptual amino acid sequence encoded by the open reading frame consisted of 1,251 amino acids (FIG. 15B) with an estimated pI of 5.92, a predicted molecular mass of 134,710 Da, and five potential *N*-linked glycosylation sites. No RGD sequence was present.

Northern blot analysis of murine embryo RNA using a 3' untranslated region probe identified a transcript band of ~4.6 kb. In this regard, 4,310 nt have been isolated by cDNA cloning, including a 3' untranslated region of 401 nt and a 5' upstream sequence of 156 nt. The apparent discrepancy between the Northern analysis result and the cDNA sequence analysis suggested that the 5' upstream sequence may include ~300 nt of additional upstream sequence. This estimate was consistent with preliminary primer extension mapping studies indicating that the 5' upstream sequence is 400-500 nt in length.

A total of 19 cysteine-rich repeats were found in domains #2 and #4 of the murine LTBP-like (LTBP-3) polypeptide. Thirteen were EGF-like and 11/13 contained the calcium binding consensus sequence. This consensus was derived from an analysis of 154 EGF-CB repeats in 23 different proteins and from structural analyses of the EGF-CB repeat, both bound and unbound to calcium ion (Selander-Sunnerhagen *et al.*, 1992). Variations on the consensus have been noted previously and one of these, D-L-N/D-E-C_1, was identified in the third EGF-like repeat of domain #4. In addition, a potential calcium binding sequence which has not previously been reported (E-T-N/D-E-C_1) was identified in the first EGF-like repeat of domain #4. Ten of thirteen EGF-CB repeats also contained a second consensus sequence which represents a recognition sequence for an Asp/Asn hydroxylase that co- and post-translationally modifies D/N residues (Stenflo *et al.*, 1987; Gronke *et al.*, 1989).

Although about one-half the size, the deduced polypeptide was organized like fibrillin-1 in that it consisted of a signal peptide followed by 5 structurally distinct domains, *i.e.*, two domains with numerous EGF-like, EGF-CB and Fib repeats and a third with a proline-rich sequence (Pereira *et al.*, 1993). However, comparison of each of these domains using the GAP and BESTFIT programs (Genetics Computer Group) has revealed a low level of amino acid homology of only 27% over the five structural domains shared by the deduced murine polypeptide and human fibrillin-2. These values are low for a putative fibrillin family member because fibrillin-1 and fibrillin-2 share ~50% identity (Zhang *et al.*, 1994).

A search of available databases revealed that the deduced murine polypeptide was most similar to the human and rat latent TGF-β binding proteins (Kanzaki *et al.*, 1990; Tsuji *et al.*, 1990). In this regard LTBP was found to be similar to fibrillin in that it could also be divided into five structurally distinct domains (FIG. 15A, FIG. 15B, and FIG. 15C). These include a relatively short domain downstream of the signal peptide with a net basic charge (amino acids 21-33, est. pI, 11.14); a domain consisting of EGF-like, EGF-CB, TGF-bp, and Fib motifs plus a proline-rich and glycine-rich sequence (amino acids 34-407); a proline-rich domain (amino acids 408-545); a large, domain consisting of EGF-CB, TGF-bp, and TGF-bp-like repeat motifs (amino acids 546-1379); and a relatively short domain at the carboxy terminus (amino acids 1380-1394). Amino acid sequence comparison of the deduced murine and human polypeptides shows 60% identity for domain #1, 52% identity for domain #2, 30% identity for domain #3, 43% identity for domain #4, and 7% identity for domain #5. The average identity over the five domains shared by the murine polypeptide and human

LTBP was 38.4%. Significantly, cysteine residues in both polypeptide sequences were highly conserved.

The fibrillins are exclusively expressed by connective cells in developing tissues (Zhang *et al.*, 1994), whereas LTBP should be expressed along with TGF-β by both epithelial and connective cells (Tsuji *et al.*, 1990). The structural homology data therefore predict that the murine LTBP-3 gene shown in FIG. 15B should be expressed by both epithelial and connective tissue cells. Tissue *in situ* hybridization was used to test this hypothesis.

An overview of the expression pattern as determined by tissue *in situ* hybridization is presented in FIG. 17A, FIG. 17B, FIG. 17C, and FIG. 17D. Approximate mid-sagittal sections of normal murine embryos at days 8.5-9.0, 13.5 and 16.5 *p.c.* of development were hybridized with a ^{35}S-labeled single stranded normal sense riboprobe from the same cDNA construct was used. At day 8.5-9.0 of development, intense gene expression was observed in the mesometrial and anti-mesometrial uterine tissues, ectoplacental cone, placenta, placental membranes. The transcript appeared to be widely expressed in murine embryo mesenchymal/connective tissue compartments, including the facial mesenchyme, at days 8.5-9.0, 13.5 and 16.5 of development. Particularly intense expression of the transcript was noted in the liver.

Microscopy of day 8.5-9.0 embryos confirmed the widespread expression of the murine gene by mesenchymal cells. Significant expression of the transcript by cells of the developing central nervous system, somites and

cardiovascular tissue (myocardium plus endocardium) was also observed.

Microscopy of day 13.5 and day 16.5 embryos demonstrated expression of the murine gene by skeletal muscle cells and by cells involved in intramembranous and endochondral bone formation. The transcript was expressed by osteoblasts and by periosteal cells of the calvarium, mandible and maxilla. The transcript was also identified in both cartilage and bone of the lower extremity. A positive signal was detected in perichondrial cells and chondrocytes (proliferating > mature > hypertrophic) of articular cartilage, the presumptive growth plate, and the cartilage model within the central canal. The positive signal was also expressed by blood vessel endothelial cells within the mid-diaphysis, and the surrounding muscle cells (FIG. 18A, FIG. 18B, FIG. 18C, FIG. 18D, FIG. 18E, FIG. 18F, FIG. 19G, FIG. 18H, FIG. 18I, FIG. 18J, FIG. 18K, FIG. 18L, FIG. 18M, FIG. 18N, FIG. 18O, and FIG. 18P).

Respiratory epithelial cells lining developing small airways and connective tissue cells in the pulmonary interstitium expressed the murine transcript, as did myocardial cells (atria and ventricles) and endocardial cushion tissue. Cells within the walls of large arteries also expressed the transcript. Expression of the murine gene was identified in several organs of the alimentary system, including the tongue, esophagus, stomach, small and large intestine, pancreas and liver. Mucosal epithelial cells lining the upper and lower digestive tract plus the smooth muscle and connective tissue cells found in the submucosa expressed the transcript, as did acinar cells of the exocrine pancreas. Despite the high level of transcript expression in the liver, these results suggest both cell populations express the LTBP-3 transcript.

In the kidney, expression above the basal level was observed in cells of developing nephrons, the ureteric bud, kidney blastema and the kidney interstitium. In the skin, epidermal and adnexal keratinocytes, dermal connective tissue cells, and brown fat cells within the dorsal subcutis expressed the murine transcript. In the central and peripheral nervous systems, ganglion cells within the cerebrum, brainstem, spinal cord, and peripheral nerves expressed the murine transcript. The transcript was also intensely expressed by cells of the developing murine retina.

Thus, the murine gene is widely expressed by both epithelial and connective tissue cells, a pattern that would be expected for a latent TGF-β binding protein. Three final observations argue that the LTBP-like (LTBP-3) sequence presented in FIG. 25 is not simply the murine homologue of human LTBP. First, domain #4 of the murine LTBP-like (LTBP-3) sequence has a smaller number of EGF-like repeat motifs than human and rat LTBP (8 versus 11). Second, portions of the human and rat LTBP-like coding sequence were characterized and found to share ~90% identity with human and rat LTBP but only 65% identity with the murine LTBP-like gene. Third, the human LTBP and LTBP-like genes are localized to separate chromosomes. Human LTBP was assigned to human chromosome 2 based on the analysis of human *x* rodent somatic cell hybrid lines (Stenman *et al.*, 1994). The present invention represents the first mapping of an LTBP gene in the murine. The human LTBP-like genes was recently localized to chromosome 11 band q12, while the murine gene was mapped to murine chromosome 19, band B (a region of conserved synteny), using several independent approaches, including fluorescent *in situ* hybridization.

The first indication of alternative splicing came from molecular cloning studies in the murine, in which

independent cDNA clones were isolated with a deletion of 51 bp from the coding sequence. PCR™/Southern blot analysis provided additional evidence that the homologous 51 bp sequence was alternatively spliced in normal murine embryo tissues.

Northern blot analysis also demonstrated that the novel fibrillin gene was also expressed in rat callus three weeks after osteotomy, after mineralization has begun. Gene expression in normal adult rat bone tissue was insignificant, which suggests that microfibrils are an important part of the bone fracture healing response. The novel fibrillin-like gene was expressed in callus as a pair of alternatively spliced transcripts. This result has been independently reproduced on three occasions. Molecular cloning of the novel fibrillin gene in both murine and rat has identified potential splice junction sites for the alternative splicing event.

MC3T3-E1 murine pre-osteoblasts were used to demonstrate that the murine gene product was capable of binding TGF-β. MC3T3-E1 cells were utilized because they synthesize and secrete TGF-β, which may act as an autocrine regulator of osteoblast proliferation (Amarnani *et al.*, 1993; Van Vlasselaer *et al.*, 1994; Lopez-Casillas *et al.*, 1994).

To determine whether or not MC3T3-E1 cells co-expressed the murine gene product of TGF-β, cells were plated on 100-mm dishes under differentiating conditions (Quarles *et al.*, 1992) and the medium was replaced twice weekly. Parallel dishes were plated and assayed for cell number and alkaline phosphatase activity, which confirmed that osteoblast differentiation was indeed taking place. Equal aliquots of total cellular RNA was prepared from these MC3T3-E1 cells after 5, 14 and 28 days in culture for Northern blot analysis. As shown in FIG. 19,

expression of the new murine gene peaked on day 14 of culture. Since MC3T3-E1 cells also show a peak in alkaline phosphatase activity on day 14 of culture (Quarles *et al.*, 1992), the results suggest for the first time that LTBP-2 gene expression is an early marker of osteoblast differentiation.

C. DISCUSSION

This study reports the molecular cloning of a novel LTBP-like gene that contains numerous EGF-like repeats. Northern analysis indicates that the gene encodes a single transcript of ~4.6 kb in murine embryo tissues. The deduced amino acid sequence of the murine gene product appears to be a secreted polypeptide of 1,251 amino acids. Although it is similar to fibrillin, the overall structural organization and expression pattern of this gene product most resembles LTBP, a latent TGF-β binding protein that was originally isolated and characterized by Heldin and co-workers (Kanzaki *et al.*, 1990). Several observations strongly suggest that LTBP and the murine LTBP-like gene product are therefore derived from related but distinct genetic loci. First, LTBP and the LTBP-like coding sequence share ~40% identity and differences exist in the number of EGF-CB repeats in the deduced polypeptide sequence of the two molecules. Second, a portion of the murine LTBP gene has been cloned and shown to share ~90% identity with human and rat LTBP. Conversely, portions of the human and rat LTBP-like genes have been cloned and shown to share ~90% identity with the murine LTBP-like gene. Third, LTBP and the LTBP-like gene reside on different human chromosomes (Stenman *et al.*, 1994). Taken together, these data suggest that a family of at least two LTBP genes exists.

Similarities in the structural organization of LTBP-1 and the fibrillin-1 and fibrillin-2 polypeptides have

been noted previously (Pereira *et al.*, 1993; Zhang *et al.*, 1994; Taipale *et al.*, 1994). For example, LTBP-1 and the fibrillins are all secreted extracellular matrix constituents. Moreover, each polypeptide can be organized into five domains, two of which consists predominantly of EGF-CB and TGF-bp repeat motifs. LTBP-1 and fibrillin-1 also share a domain that is proline-rich, and LTBP possesses an 8-cysteine repeat previously referred to as the "Fib motif" because it was assumed to be unique to fibrillin (Pereira *et al.*, 1993). These similarities likely explain the initial isolation and cloning of the LTBP-2 PCR™ product, especially since the human oligonucleotide primers used to initially amplify murine cDNA were designed to direct the synthesis of an EGF-CB repeat in domain #4.

Another point of distinction between LTBP-2 and fibrillin concerns the spacing of conserved cysteines C4 and C5 in EGF-like repeats. Fibrillin-1 and fibrillin-2 each contain >50 such repeats, and in every one the spacing is C_4-X-C_5. While this pattern is repeated in a majority of the EGF-like repeats in LTBP-1 and LTBP-2, both genes also contain repeats with the spacing C_4-X-X-C_5. Although the significance of this observation is unclear, variation in the number of amino acids between C_4 and C_5 would not be expected to alter the function of the EGF-like repeat. Mature EGF is a 48 amino acid secreted polypeptide consisting of two subdomains that have few interdomain contacts (Engel, 1989; Davis, 1990). The larger NH_2-terminal subdomain consists of residues 1-32 and is stabilized by a pair of disulfide bonds (C_1-C_3 and C_2-C_4), whereas the smaller COOH-terminal subdomain (amino acids 33-48) is stabilized by a single disulfide bond (C_5-C_6). The COOH-terminal subdomain has a highly conserved conformation that only is possible if certain residues and the distances between them are well conserved, while conformation-sequence requirements for the NH2-terminal

subdomain are relatively relaxed. Variation in C_4-C_5 spacing would not be expected to alter conformation because these residues do not normally form a disulfide bond and the spacing variation occurs at the interface of subdomains that would not be predicted to interact. The cloning of additional genes will decide whether variation in C_4-C_5 spacing is a reliable discriminator between members of the LTBP and fibrillin gene families.

The LTBP-2 gene is expressed more widely during development than fibrillin-1 or fibrillin-2. Studies in developing murine tissues have shown that the *Fbn-1* gene is expressed by mesenchymal cells of developing connective tissue, whereas the murine LTBP-like gene is intensely expressed by epithelial, parenchymal and stromal cells. Earlier reports have suggested that TGF-β plays a role in differentiation and morphogenesis during murine development (Lyons and Moses, 1990), when TGF-β is produced by epithelial, parenchymal and stromal cells. Tsuji *et al.*, (1990) and others have suggested that the expression of TGF-β binding proteins should mirror that of TGF-β itself; the expression pattern of the LTBP-2 gene over the course of murine development is consistent with this expectation. However, the LTBP-2 gene may not be completely co-regulated with TGF-β. TGF-β gene and protein expression during murine development has been surveyed extensively (Heine *et al.*, 1987; Lehnert and Akhurst, 1988; Pelton *et al.*, 1989; Pelton *et al.*, 1990a,b; Millan *et al.*, 1991); these studies have not identified expression by skeletal muscle cells, chondrocytes, hepatocytes, ganglion cells, mucosal cells lining the gut, and epithelial cells of developing nephrons. It is conceivable that the LTBP-2 molecule has

an additional function in certain connective tissues besides targeting TGF-β.

The binding properties of the LTBP-2 gene product are under investigation. Formally, the LTBP-2 polypeptide may bind a specific TGF-β isoform, another member of the TGF-β superfamily (*e.g.*, a bone morphogenetic protein, inhibin, activin, or Mullerian inhibiting factor), or a growth factor unrelated to TGF-β. Anti-peptide antibodies to the murine LTBP-2 polypeptide have been generated and osteoblast cell lines that express the molecule at relatively high levels have been identified. Studies with these reagents suggest that LTBP-2 assembles intracellularly into large latent complexes with a growth factor that is being characterized by immunological methods.

The presence of dibasic amino acids in the LTBP-2 sequence suggests that it may undergo cell- and tissue-specific proteolysis. TGF-β regulates extracellular matrix production by suppressing matrix degradation (through a decrease in the expression of proteases such as collagenase, plasminogen activator, and stromelysin plus an increase in the expression of proteinase inhibitors such as plasminogen activator inhibitor-1 and tissue inhibitor of metalloproteinase-1) and by stimulating matrix macromolecule synthesis (for recent review, see Lyons and Moses, 1990; Massague, 1990; Laiho and Keski-Oja, 1992; Miyazono *et al.*, 1992). Conversely, production of extracellular matrix has been shown to down regulate TGF-β gene expression (Streuli *et al.*, 1993). TGF-β may therefore regulate extracellular matrix production through a sophisticated feedback loop that influences the expression of a relatively large number of genes. LTBP-1 and LTBP-2 may contribute to this regulation by facilitating the assembly and secretion of large latent growth factor complexes and then targeting

the complex to specific connective tissues (Taipale *et al.*, 1994).

If LTBP-3 is like LTBP-1, it has the potential to function as a secreted, extracellular structural protein. As demonstrated here, domain #1 of LTBP-3 appears to be a unique sequence that likely has a globular conformation. Domain #1 also is highly basic and may facilitate LTBP-2 binding to acidic molecules (*e.g.*, acidic proteoglycans) within the extracellular space. Sequences rich in basic amino acids have also been shown to function as endoproteolytic processing signals for several peptide hormones (Barr, 1991; Steiner *et al.*, 1992). It is possible, therefore, that the NH_2-terminus of LTBP-3 is proteolytically processed in a tissue-specific manner. Domains #2 and #4 consist of consecutive cysteine-rich repeats, the majority of which are of the EGF-CB type. Besides binding calcium (Corson *et al.*, 1993), these repeats may provide LTBP-3 with regions conformation capable of interacting with other matrix macromolecules (Engel, 1989). Domain #3 is proline rich and may be capable of bending (or functioning like a hinge) in three-dimensional space (MacArthur and Thornton, 1991). (In this regard, domain #2 is of interest because it has a similar stretch of 135 amino acids that is both proline- and glycine-rich. Since glycine-rich sequences are also thought to be capable of bending or functioning like a hinge in three-dimensional space, this amino acid sequence may interrupt the extended conformation of domain #2, thereby providing it with a certain degree of flexibility in three-dimensional space.) Domain #5 also appears to be a unique sequence having a globular conformation. The absence of a known cell attachment motif may indicate that, in contrast to LTBP-1, the LTBP-3 molecule may have a more limited role in the extracellular matrix (*i.e.*, that of a structural protein)

in addition to its ability to target latent TGF-β complexes to specific connective tissues.

MC3T3-E1 pre-osteoblasts co-express LTBP-3 and TGF-β1 and these proteins form a complex in the culture medium. These results are particularly interesting because bone represents one of the largest known repositories of latent TGF-β (200 μg/kg bone; Seyedin *et al.*, 1986 and 1987), and because this growth factor plays a critical role in the determination of bone structure and function. For example, TGF-β is thought to (i) provide a powerful stimulus to bone formation in developing tissues, (ii) function as a possible "coupling factor" during bone remodeling (a process that coordinates bone resorption and formation), and (iii) exert a powerful bone inductive stimulus following fracture. Activation of the latent complex may be an important step governing TGF-β effects, and LTBP may modulate the activation process (*e.g.*, it may "protect" small latent complexes from proteolytic attack).

Expression of large latent TGF-β complexes bearing LTBP may be physiologically relevant to, *i.e.*, may be part of the mechanism of, the pre-osteoblast → osteoblast differentiation cascade. This is based on the evidence that MC3T3-E1 cells express large latent TGF-β1 complexes bearing LTBP-2 precisely at the time of transition from the pre-osteoblast to osteoblast phenotype (~day 14 in culture, or, at the onset of alkaline phosphatase expression; see Quarles *et al.*, 1992). The organ culture model, for example, likely is comprised of differentiated osteoblasts but few bond progenitors, making it a difficult model at best in which to study the differentiation cascade (Dallas *et al.*, 1984). It is also well known that MG63, ROS17/2.8 and UMR 106 cells are rapidly dividing *and* they express the osteoblast phenotype. Thus, these osteoblast-like cell lines do not

show the uncoupling of cell proliferation and cell differentiation that characterizes the normal (physiologically relevant) pre-osteoblast → osteoblast transition (Gerstenfeld *et al.*, 1984; Stein and Lian, 1993). Therefore, the production of small versus large latent TGF-β complexes may be associated with specific stages in the maturation of bone cells.

LTBP-3 may bind calcium, since EGF-CB repeats have been shown to mediate high affinity calcium binding in LTBP-1 and other proteins (Colosetti *et al.*, 1993). Calcium binding, in turn, may contribute to molecular conformation and the regulation of its interactions with other molecules. The presence of dibasic amino acids suggests that LTBP-3 may also undergo cell- and tissue-specific proteolysis. TGF-β regulates extracellular matrix production by suppressing matrix degradation (through a decrease in the expression of proteases such as collagenase, plasminogen activator, and stromelysin plus an increase in the expression of proteinase inhibitors such as plasminogen activator inhibitor-1 and tissue inhibitor of metalloproteinase-1) and by stimulating matrix macromolecule synthesis (for recent reviews, see Lyons and Moses, 1990; Massague, 1990; Laiho and Keski-Oja, 1992; and Miyanzono *et al.*, 1993). Conversely, production of extracellular matrix has been shown to down regulate TGF-β gene expression (Streuli *et al.*, 1993). TGF-β may therefore regulate extracellular matrix production through a sophisticated feedback loop that influences the expression of a relatively large number of genes. LTBP-1, LTBP-2, and LTBP-3 may contribute to this regulation by facilitating the assembly and secretion of large latent growth factor

complexes and then targeting the complex to specific connective tissues (Taipale *et al.*, 1994).

EXAMPLE XVI

PREPARATION OF ANTIBODIES AGAINST THE LTBP-3 GENE PRODUCT

An affinity-purified antibody (#274) capable of immunoprecipitating was prepared against the murine LTBP-3 gene product. A full-length murine cDNA was assembled into the pcDNA3 mammalian expression vector (Invitrogen) and expressed following transient transfection of 293T cells. Nascent polypeptides, radiolabeled by addition of ^{35}S Cys to the medium of transfected cells, were immunoprecipitated using affinity-purified antibody #274. As shown in FIG. 20, the new murine polypeptide was estimated to be 180-190 kDa. To ensure the specificity of #274 binding, we showed that preincubation with 10 μg of synthetic peptide blocks immunoprecipitation of the 180-190 kDa band.

Finally, MC3T3-E1 cells were cultured for 7 days under differentiating conditions and double-labeled with 30 $\mu Ci/ml$ ^{35}S cysteine and ^{35}S methionine in deficient media. Radiolabeled media was dialyzed into cold PBS with protease inhibitors. Aliquots of the dialyzed medium sample (10^6 incorporated CPM) were analyzed by a combined immunoprecipitation/Western analysis protocol. The murine polypeptide was clearly and reproducibly secreted by MC3T3-E1 cells, migrating under reducing conditions as a single band of 180-190 kDa (FIG. 21). Consistent with the results of previous studies (*e.g.*, Miyazono *et al.*, 1988; Dallas *et al.*, 1994; Moren *et al.*, 1994), bands of 70 and 50 kDa corresponding to the TGF-β1 precursor were co-immunoprecipitated with the 180 kDa LTBP-3 protein. Weak bands of 40 and 12 kDa were also identified in experiments in which only immunoprecipitation was performed. The latter were not

included in FIG. 21 because they migrated within that portion of the gel included in the Western analysis. Protein bands of 70-12.5 kDa are not variant forms of LTBP-3; FIG. 20 demonstrates that LTBP-3 migrates as a single band of 180-190 kDa following transient transfection of 293T cells, which fail to make TGF-β1. By immunoprecipitation, a unique band consistent with monomeric mature TGF-β1 was found in the LTBP-2 immunoprecipitate. Antibody #274 is incapable of binding TGF-β1 as determined by radioimmunoassay using commercially available reagents (R&D Systems) and the manufacturer's suggested protocols. These results have been reproduced in 6 independent experiments which utilized 3 separate lots of MC3T3-E1 medium. Thus the new murine LTBP-3 polypeptide binds TGF-β *in vitro*.

EXAMPLE XVII

ISOLATION OF A GENE ENCODING MURINE LTBP-2

In addition to determining the DNA and corresponding polypeptide sequence of the murine LTBP-3 gene, the murine LTBP-2 gene was also cloned and sequenced.

The complete cDNA nucleotide sequence for murine LTBP-2 is shown in FIG. 27 (SEQ ID NO:17). The deduced amino acid sequence is shown in FIG. 28 (SEQ ID NO:18).

EXAMPLE XVIII

EXPRESSION OF RECOMBINANT TYPE II COLLAGEN

The *Pichia* Expression Kit (Invitrogen, Inc.) may be used to prepare recombinant type II collagen. This kit, based on the methylotrophic yeast, *Pichia pastoris*, allows high-level expression of recombinant protein in an easy-to-use relatively inexpensive system. In the absence of the preferred carbon source, glucose, *P.*

pastoris utilizes methanol as a carbon source. The *AOX1* promoter controls the gene that codes for the expression of the enzyme alcohol oxidase, which catalyzes the first step in the metabolism of methanol. This promoter, which is induced by methanol, has been characterized and incorporated into a series of *Pichia* expression vectors. This feature of *Pichia* has been exploited to express high levels of recombinant proteins often in the range of grams per liter. Because it is eukaryotic, *P. pastoris* utilizes posttranslational modification pathways that are similar to those used by mammalian cells. This implies that the recombinant type II collagen will be glycosylated and will contain disulfide bonds.

The inventors contemplate the following particular elements to be useful in the expression of recombinant type II collagen: the DNA sequence of human type II collagen (SEQ ID NO:11) (Lee *et al.*, 1989); rat type II collagen (SEQ ID NO:13) (Michaelson, *et al.*, 1994); and/or mouse type II collagen (SEQ ID NO:15) (Ortman, *et al.*, 1994). As other sources of DNA sequences encoding type II collagen are available, these three are examples of many sequence elements that may be useful in the present invention.

For preparation of a recombinant type II collagen, the native type II collagen cDNA is modified by the addition of a commercially available epitope tag (the HA epitope, Pharmacia, LKB Biotechnology, Inc.). Such fragments may be readily prepared by, for example, directly synthesizing the fragment by chemical means, by application of nucleic acid reproduction technology, such as the PCR technology of U.S. Patent 4,603,102 (herein incorporated by reference) or by introducing selected sequences into recombinant vectors for recombinant production. (PCR™ is a registered trademark of Hoffmann-LaRoche, Inc.). This is followed by cloning into the

Pichia expression vector. The resulting plasmid is characterized by DNA sequence analysis, linearized by digestion with *Not*I, and spheroplasts will be prepared and transformed with the linearized construct according to the manufacturer's recommendations.

Transformation facilitates a recombination event *in vivo* between the 5' and 3' *AOX1* sequences in the *Pichia* vector and those in the *Pichia* genome. The result is the replacement of *AOX1* with the gene of interest.

Transformants are then plated on histidine-deficient media, which will select for successfully transformed cells. Transformants are further selected against slow growth on growth media containing methanol. Positive transformants are grown for 2 days in liquid culture and then for 2-6 days in broth that uses methanol as the sole carbon source. Protein expression is evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western hybridization using a commercially available polyclonal antisera to the HA epitope (Pharmacia).

Recombinant type II collagen protein can be purified according to the manufacturer's recommendations, dialyzed against double distilled, deionized water and lyophilized in 10 mg aliquots. The aliquots are sterilized and used as implant material for the osteoconductive matrices.

* * *

All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations

may be applied to the composition, methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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Zhang *et al.*, *J. Cell Biol.*, 124:855-863, 1994.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

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The Wolverine Tower, Room 2071

(C) CITY: Ann Arbor

(D) STATE: Michigan

(E) COUNTRY: United States of America

(F) POSTAL (ZIP) CODE: 48109-1280

(ii) INVENTORS: BONADIO, Jeffrey
ROESSLER, Blake J.
GOLDSTEIN, Steven A.
LIN, Wushan

(iii) TITLE OF INVENTION: METHODS AND COMPOSITIONS FOR STIMULATING BONE CELLS

(iv) NUMBER OF SEQUENCES: 18

(v) CORRESPONDENCE ADDRESS:

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(C) CITY: Houston

(D) STATE: Texas

(E) COUNTRY: United States of America

(F) ZIP: 77210

(vi) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS/ASCII

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vii) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: UNKNOWN
(B) FILING DATE: CONCURRENTLY HEREWITH
(C) CLASSIFICATION: UNKNOWN

(viii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/316,650
(B) FILING DATE: 30-SEP-1994
(C) CLASSIFICATION: UNKNOWN

(A) APPLICATION NUMBER: US 08/199,780
(B) FILING DATE: 18-FEB-1994
(C) CLASSIFICATION: UNKNOWN

(ix) ATTORNEY/AGENT INFORMATION:
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(B) REGISTRATION NUMBER: 32,165
(C) REFERENCE/DOCKET NUMBER: UMIC009P--

(x) TELECOMMUNICATION INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 417 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Met Ile Pro Gly Asn Arg Met Leu Met Val Val Leu Leu Cys Gln Val
1               5                   10                  15

Leu Leu Gly Gly Ala Thr Asp Ala Ser Leu Met Pro Glu Thr Gly Lys
            20                  25                  30

Lys Lys Val Ala Glu Ile Gln Gly His Ala Gly Gly Arg Arg Ser Gly
        35                  40                  45

Gln Ser His Glu Leu Leu Arg Asp Phe Glu Ala Thr Leu Leu Gln Met
    50                  55                  60

Phe Gly Leu Arg Arg Arg Pro Gln Pro Ser Lys Ser Ala Val Ile Pro
65                  70                  75                  80

Asp Tyr Met Ser Asp Leu Tyr Arg Leu Gln Ser Gly Glu Glu Glu Glu
                85                  90                  95

Glu Glu Gln Ser Gln Gly Thr Gly Leu Glu Tyr Pro Glu Arg Pro Ala
            100                 105                 110
```

```
Ser Ser Ala Asn Thr Val Ser Ser Phe His His Glu Glu His Leu Glu
        115                 120                 125

Asn Ile Pro Gly Thr Ser Glu Ser Ser Ala Phe Arg Phe Phe Phe Asn
    130                 135                 140

Leu Ser Ser Ile Pro Glu Asn Glu Val Ile Ser Ser Ala Glu Leu Arg
145                 150                 155                 160

Leu Phe Arg Glu Gln Val Asp Gln Gly Pro Asp Trp Glu Gln Gly Phe
                165                 170                 175

His Arg Met Asn Ile Tyr Glu Val Met Lys Pro Pro Ala Glu Met Val
            180                 185                 190

Pro Gly His Leu Ile Thr Arg Leu Leu Asp Thr Ser Leu Val Arg His
        195                 200                 205

Asn Val Thr Arg Trp Glu Thr Phe Asp Val Ser Pro Ala Val Leu Arg
    210                 215                 220

Trp Thr Arg Glu Lys Gln Pro Asn Tyr Gly Leu Ala Ile Glu Val Thr
225                 230                 235                 240
```

```
His Leu His Gln Thr Arg Thr His Gln Gly Gln His Val Ser Ile Ser
                245                 250                 255

Arg Ser Leu Pro Gln Gly Ser Gly Asn Trp Ala Gln Leu Arg Pro Leu
            260                 265                 270

Leu Val Thr Phe Gly His Asp Gly Arg Gly His Thr Leu Thr Arg Arg
        275                 280                 285

Ser Ala Lys Arg Ser Pro Lys His His Pro Gln Arg Ser Ser Lys Lys
    290                 295                 300

Asn Lys Asn Cys Arg Arg His Ser Leu Tyr Val Asp Phe Ser Asp Val
305                 310                 315                 320

Gly Trp Asn Asp Trp Ile Val Ala Pro Pro Gly Tyr Gln Ala Phe Tyr
                325                 330                 335

Cys His Gly Asp Cys Pro Phe Pro Leu Ala Asp His Leu Asn Ser Thr
            340                 345                 350

Asn His Ala Ile Val Gln Thr Leu Val Asn Ser Val Asn Ser Ser Ile
        355                 360                 365
```

```
Pro Lys Ala Cys Cys Val Pro Thr Glu Leu Ser Ala Ile Ser Met Leu
    370                 375                 380

Tyr Leu Asp Glu Tyr Asp Lys Val Val Leu Lys Asn Tyr Gln Glu Met
385                 390                 395                     400

Val Val Glu Gly Cys Gly Cys Arg Tyr Pro Tyr Asp Val Pro Asp Tyr
                405                 410                 415

Ala
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 3753 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..3753

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
ATG CGC CAG GCC GCA TTG GGG CTG CTG GCA CTA CTC CTG CTG GCG CTG      48
Met Arg Gln Ala Ala Leu Gly Leu Leu Ala Leu Leu Leu Leu Ala Leu
 1               5                  10                  15

CTG GGC CCC GGC GGC CGA GGG GTG GGC CGG CCG GGC AGC GGG GCA CAG      96
Leu Gly Pro Gly Gly Arg Gly Val Gly Arg Pro Gly Ser Gly Ala Gln
            20                  25                  30

GCG GGG GCG GGG CGC TGG GCC CAA CGC TTC AAG GTG GTC TTT GCG CCT     144
Ala Gly Ala Gly Arg Trp Ala Gln Arg Phe Lys Val Val Phe Ala Pro
        35                  40                  45

GTG ATC TGC AAG CGG ACC TGT CTG AAG GGC CAG TGT CGG GAC AGC TGT     192
Val Ile Cys Lys Arg Thr Cys Leu Lys Gly Gln Cys Arg Asp Ser Cys
    50                  55                  60
```

```
CAG CAG GGC TCC AAC ATG ACG CTC ATC GGA GAG AAC GGC CAC AGC ACC      240
Gln Gln Gly Ser Asn Met Thr Leu Ile Gly Glu Asn Gly His Ser Thr
 65                  70                  75                  80

GAC ACG CTC ACC GGT TCT GCC TTC CGC GTG GTG GTG TGC CCT CTA CCC      288
Asp Thr Leu Thr Gly Ser Ala Phe Arg Val Val Val Cys Pro Leu Pro
                 85                  90                  95

TGC ATG AAC GGT GGC CAG TGC TCT TCC CGA AAC CAG TGC CTG TGT CCC      336
Cys Met Asn Gly Gly Gln Cys Ser Ser Arg Asn Gln Cys Leu Cys Pro
            100                 105                 110

CCG GAT TTC ACG GGG CGC TTC TGC CAG GTG CCT GCT GCA GGA ACC GGA      384
Pro Asp Phe Thr Gly Arg Phe Cys Gln Val Pro Ala Ala Gly Thr Gly
        115                 120                 125

GCT GGC ACC GGG AGT TCA GGC CCC GGC TGG CCC GAC CGG GCC ATG TCC      432
Ala Gly Thr Gly Ser Ser Gly Pro Gly Trp Pro Asp Arg Ala Met Ser
    130                 135                 140

ACA GGC CCG CTG CCG CCC CTT GCC CCA GAA GGA GAG TCT GTG GCT AGC      480
Thr Gly Pro Leu Pro Pro Leu Ala Pro Glu Gly Glu Ser Val Ala Ser
145                 150                 155                 160
```

```
AAA CAC GCC ATT TAC GCG GTG CAG GTG ATC GCA GAT CCT CCC GGG CCG      528
Lys His Ala Ile Tyr Ala Val Gln Val Ile Ala Asp Pro Pro Gly Pro
                165                 170                 175

GGG GAG GGT CCT CCT GCA CAA CAT GCA GCC TTC TTG GTG CCC CTG GGG      576
Gly Glu Gly Pro Pro Ala Gln His Ala Ala Phe Leu Val Pro Leu Gly
            180                 185                 190

CCA GGA CAA ATC TCG GCA GAA GTG CAG GCT CCG CCC CCC GTG GTG AAC      624
Pro Gly Gln Ile Ser Ala Glu Val Gln Ala Pro Pro Pro Val Val Asn
        195                 200                 205

GTG CGT GTC CAT CAC CCT CCT GAA GCT TCC GTT CAG GTG CAC CGC ATC      672
Val Arg Val His His Pro Pro Glu Ala Ser Val Gln Val His Arg Ile
    210                 215                 220

GAG GGG CCG AAC GCT GAA GGC CCA GCC TCT TCC CAG CAC TTG CTG CCG      720
Glu Gly Pro Asn Ala Glu Gly Pro Ala Ser Ser Gln His Leu Leu Pro
225                 230                 235                 240

CAT CCC AAG CCC CCG CAC CCG AGG CCA CCC ACT CAA AAG CCA CTG GGC      768
His Pro Lys Pro Pro His Pro Arg Pro Pro Thr Gln Lys Pro Leu Gly
                245                 250                 255
```

```
CGC TGC TTC CAG GAC ACA TTG CCC AAG CAG CCT TGT GGC AGC AAC CCT      816
Arg Cys Phe Gln Asp Thr Leu Pro Lys Gln Pro Cys Gly Ser Asn Pro
            260                 265                 270

TTG CCT GGC CTT ACC AAG CAG GAA GAT TGC TGC GGT AGC ATC GGT ACT      864
Leu Pro Gly Leu Thr Lys Gln Glu Asp Cys Cys Gly Ser Ile Gly Thr
        275                 280                 285

GCC TGG GGA CAA AGC AAG TGT CAC AAG TGC CCA CAG CTT CAG TAT ACA      912
Ala Trp Gly Gln Ser Lys Cys His Lys Cys Pro Gln Leu Gln Tyr Thr
    290                 295                 300

GGG GTG CAG AAG CCT GTA CCT GTA CGT GGG GAG GTG GGT GCT GAC TGC      960
Gly Val Gln Lys Pro Val Pro Val Arg Gly Glu Val Gly Ala Asp Cys
305                 310                 315                 320

CCC CAG GGC TAC AAG AGG CTC AAC AGC ACC CAC TGC CAG GAT ATC AAC     1008
Pro Gln Gly Tyr Lys Arg Leu Asn Ser Thr His Cys Gln Asp Ile Asn
                325                 330                 335

GAA TGT GCG ATG CCC GGG AAT GTG TGC CAT GGT GAC TGC CTC AAC AAC     1056
Glu Cys Ala Met Pro Gly Asn Val Cys His Gly Asp Cys Leu Asn Asn
            340                 345                 350
```

```
CCT GGC TCT TAT CGC TGT GTC TGC CCG CCC GGT CAT AGC TTG GGT CCC    1104
Pro Gly Ser Tyr Arg Cys Val Cys Pro Pro Gly His Ser Leu Gly Pro
        355                 360                 365

CTC GCA GCA CAG TGC ATT GCC GAC AAA CCA GAG GAG AAG AGC CTG TGT    1152
Leu Ala Ala Gln Cys Ile Ala Asp Lys Pro Glu Glu Lys Ser Leu Cys
    370                 375                 380

TTC CGC CTT GTG AGC ACC GAA CAC CAG TGC CAG CAC CCT CTG ACC ACA    1200
Phe Arg Leu Val Ser Thr Glu His Gln Cys Gln His Pro Leu Thr Thr
385                 390                 395                 400

CGC CTA ACC CGC CAG CTC TGC TGC TGT AGT GTG GGT AAA GCC TGG GGT    1248
Arg Leu Thr Arg Gln Leu Cys Cys Cys Ser Val Gly Lys Ala Trp Gly
                405                 410                 415

GCC CGG TGC CAG CGC TGC CCG GCA GAT GGT ACA GCA GCC TTC AAG GAG    1296
Ala Arg Cys Gln Arg Cys Pro Ala Asp Gly Thr Ala Ala Phe Lys Glu
            420                 425                 430

ATC TGC CCC GGC TGG GAA AGG GTA CCA TAT CCT CAC CTC CCA CCA GAC    1344
Ile Cys Pro Gly Trp Glu Arg Val Pro Tyr Pro His Leu Pro Pro Asp
        435                 440                 445
```

```
GCT CAC CAT CCA GGG GGA AAG CGA CTT CTC CCT CTT CCT GCA CCC GAC      1392
Ala His His Pro Gly Gly Lys Arg Leu Leu Pro Leu Pro Ala Pro Asp
    450                 455                 460

GGG CCA CCC AAA CCC CAG CAG CTT CCT GAA AGC CCC AGC CGA GCA CCA      1440
Gly Pro Pro Lys Pro Gln Gln Leu Pro Glu Ser Pro Ser Arg Ala Pro
465                 470                 475                 480

CCC CTC GAG GAC ACA GAG GAA GAG AGA GGA GTG ACC ATG GAT CCA CCA      1488
Pro Leu Glu Asp Thr Glu Glu Glu Arg Gly Val Thr Met Asp Pro Pro
                485                 490                 495

GTG AGT GAG GAG CGA TCG GTG CAG CAG AGC CAC CCC ACT ACC ACC ACC      1536
Val Ser Glu Glu Arg Ser Val Gln Gln Ser His Pro Thr Thr Thr Thr
            500                 505                 510

TCA CCC CCC CGG CCT TAC CCA GAG CTC ATC TCT CGC CCC TCC CCA CCT      1584
Ser Pro Pro Arg Pro Tyr Pro Glu Leu Ile Ser Arg Pro Ser Pro Pro
        515                 520                 525

ACC TTC CAC CGG TTC CTG CCA GAC TTG CCC CCA TCC CGA AGT GCA GTG      1632
Thr Phe His Arg Phe Leu Pro Asp Leu Pro Pro Ser Arg Ser Ala Val
    530                 535                 540
```

```
GAG ATC GCC CCC ACT CAG GTC ACA GAG ACC GAT GAG TGC CGA TTG AAC    1680
Glu Ile Ala Pro Thr Gln Val Thr Glu Thr Asp Glu Cys Arg Leu Asn
545                 550                 555                 560

CAG AAT ATC TGT GGC CAT GGA CAG TGT GTG CCT GGC CCC TCG GAT TAC    1728
Gln Asn Ile Cys Gly His Gly Gln Cys Val Pro Gly Pro Ser Asp Tyr
                565                 570                 575

TCC TGC CAC TGC AAC GCT GGC TAC CGG TCA CAC CCG CAG CAC CGC TAC    1776
Ser Cys His Cys Asn Ala Gly Tyr Arg Ser His Pro Gln His Arg Tyr
            580                 585                 590

TGT GTT GAT GTG AAC GAG TGC GAG GCA GAG CCC TGC GGC CCC GGG AAA    1824
Cys Val Asp Val Asn Glu Cys Glu Ala Glu Pro Cys Gly Pro Gly Lys
        595                 600                 605

GGC ATC TGT ATG AAC ACT GGT GGC TCC TAC AAT TGT CAC TGC AAC CGA    1872
Gly Ile Cys Met Asn Thr Gly Gly Ser Tyr Asn Cys His Cys Asn Arg
    610                 615                 620

GGC TAC CGC CTC CAC GTG GGT GCA GGG GGC CGC TCG TGC GTG GAC CTG    1920
Gly Tyr Arg Leu His Val Gly Ala Gly Gly Arg Ser Cys Val Asp Leu
625                 630                 635                 640
```

```
AAC GAG TGC GCC AAG CCT CAC CTG TGT GGG GAC GGT GGC TTC TGC ATC     1968
Asn Glu Cys Ala Lys Pro His Leu Cys Gly Asp Gly Gly Phe Cys Ile
                645                 650                 655

AAC TTC CCT GGT CAC TAC AAA TGC AAC TGC TAT CCT GGC TAC CGG CTC     2016
Asn Phe Pro Gly His Tyr Lys Cys Asn Cys Tyr Pro Gly Tyr Arg Leu
            660                 665                 670

AAG GCC TCC CGA CCG CCC ATT TGC GAA GAC ATC GAC GAG TGT CGC GAC     2064
Lys Ala Ser Arg Pro Pro Ile Cys Glu Asp Ile Asp Glu Cys Arg Asp
        675                 680                 685

CCT AGC ACC TGC CCT GAT GGC AAA TGT GAA AAC AAA CCT GGC AGC TTC     2112
Pro Ser Thr Cys Pro Asp Gly Lys Cys Glu Asn Lys Pro Gly Ser Phe
    690                 695                 700

AAG TGC ATC GCC TGC CAG CCT GGC TAC CGT AGC CAG GGG GGC GGG GCC     2160
Lys Cys Ile Ala Cys Gln Pro Gly Tyr Arg Ser Gln Gly Gly Gly Ala
705                 710                 715                 720

TGT CGT GAT GTC AAC GAA TGC TCC GAA GGT ACC CCC TGC TCT CCT GGA     2208
Cys Arg Asp Val Asn Glu Cys Ser Glu Gly Thr Pro Cys Ser Pro Gly
                725                 730                 735
```

```
TGG TGT GAG AAA CTT CCG GGT TCT TAC CGT TGC ACG TGT GCC CAG GGG    2256
Trp Cys Glu Lys Leu Pro Gly Ser Tyr Arg Cys Thr Cys Ala Gln Gly
            740                 745                 750

ATA CGA ACC CGC ACA GGA CGC CTC AGT TGC ATA GAC GTG GAT GAC TGT    2304
Ile Arg Thr Arg Thr Gly Arg Leu Ser Cys Ile Asp Val Asp Asp Cys
        755                 760                 765

GAG GCT GGG AAA GTG TGC CAA GAT GGC ATC TGC ACG AAC ACA CCA GGC    2352
Glu Ala Gly Lys Val Cys Gln Asp Gly Ile Cys Thr Asn Thr Pro Gly
    770                 775                 780

TCT TTC CAG TGT CAG TGC CTC TCC GGC TAT CAT CTG TCA AGG GAT CGG    2400
Ser Phe Gln Cys Gln Cys Leu Ser Gly Tyr His Leu Ser Arg Asp Arg
785                 790                 795                 800

AGC CGC TGT GAG GAC ATT GAT GAA TGT GAC TTC CCT GCG GCC TGC ATC    2448
Ser Arg Cys Glu Asp Ile Asp Glu Cys Asp Phe Pro Ala Ala Cys Ile
                805                 810                 815

GGG GGT GAC TGC ATC AAT ACC AAT GGT TCC TAC AGA TGT CTC TGT CCC    2496
Gly Gly Asp Cys Ile Asn Thr Asn Gly Ser Tyr Arg Cys Leu Cys Pro
            820                 825                 830
```

```
CTG GGT CAT CGG TTG GTG GGC GGC AGG AAG TGC AAG AAA GAT ATA GAT     2544
Leu Gly His Arg Leu Val Gly Gly Arg Lys Cys Lys Lys Asp Ile Asp
        835                 840                 845

GAG TGC AGC CAG GAC CCA GGC CTG TGC CTG CCC CAT GCC TGC GAG AAC     2592
Glu Cys Ser Gln Asp Pro Gly Leu Cys Leu Pro His Ala Cys Glu Asn
    850                 855                 860

CTC CAG GGC TCC TAT GTC TGT GTC TGT GAT GAG GGT TTC ACA CTC ACC     2640
Leu Gln Gly Ser Tyr Val Cys Val Cys Asp Glu Gly Phe Thr Leu Thr
865                 870                 875                 880

CAG GAC CAG CAT GGG TGT GAG GAG GTG GAG CAG CCC CAC CAC AAG AAG     2688
Gln Asp Gln His Gly Cys Glu Glu Val Glu Gln Pro His His Lys Lys
                885                 890                 895

GAG TGC TAC CTT AAC TTC GAT GAC ACA GTG TTC TGT GAC AGC GTA TTG     2736
Glu Cys Tyr Leu Asn Phe Asp Asp Thr Val Phe Cys Asp Ser Val Leu
            900                 905                 910

GCT ACC AAT GTC ACT CAG CAG GAA TGC TGT TGC TCT CTG GGA GCT GGC     2784
Ala Thr Asn Val Thr Gln Gln Glu Cys Cys Cys Ser Leu Gly Ala Gly
        915                 920                 925
```

TGG GGA GAC CAC TGC GAA ATC TAT CCC TGT CCA GTC TAC AGC TCA GCC 2832
Trp Gly Asp His Cys Glu Ile Tyr Pro Cys Pro Val Tyr Ser Ser Ala
930 935 940

GAA TTT CAC AGC CTG GTG CCT GAT GGG AAA AGG CTA CAC TCA GGA CAA 2880
Glu Phe His Ser Leu Val Pro Asp Gly Lys Arg Leu His Ser Gly Gln
945 950 955 960

CAA CAT TGT GAA CTA TGC ATT CCT GCC CAC CGT GAC ATC GAC GAA TGC 2928
Gln His Cys Glu Leu Cys Ile Pro Ala His Arg Asp Ile Asp Glu Cys
965 970 975

ATA TTG TTT GGG GCA GAG ATC TGC AAG GAG GGC AAG TGT GTG AAC TCG 2976
Ile Leu Phe Gly Ala Glu Ile Cys Lys Glu Gly Lys Cys Val Asn Ser
980 985 990

CAG CCC GGC TAC GAG TGC TAC TGC AAG CAG GGC TTC TAC TAC GAT GGC 3024
Gln Pro Gly Tyr Glu Cys Tyr Cys Lys Gln Gly Phe Tyr Tyr Asp Gly
995 1000 1005

AAC CTG CTG GAG TGC GTG GAC GTG GAC GAG TGC TTG GAT GAG TCT AAC 3072
Asn Leu Leu Glu Cys Val Asp Val Asp Glu Cys Leu Asp Glu Ser Asn
1010 1015 1020

```
TGC AGG AAC GGA GTG TGT GAG AAC ACG TGG CGG CTA CCG TGT GCC TGC     3120
Cys Arg Asn Gly Val Cys Glu Asn Thr Trp Arg Leu Pro Cys Ala Cys
1025                1030                1035                1040

ACT CCG CCG GCA GAG TAC AGT CCC GCA CAG GCC CAG TGT CTG ATC CCG     3168
Thr Pro Pro Ala Glu Tyr Ser Pro Ala Gln Ala Gln Cys Leu Ile Pro
                1045                1050                1055

GAG AGA TGG AGC ACG CCC CAG AGA GAC GTG AAG TGT GCT GGG GCC AGC     3216
Glu Arg Trp Ser Thr Pro Gln Arg Asp Val Lys Cys Ala Gly Ala Ser
            1060                1065                1070

GAG GAG AGG ACG GCA TGT GTA TGG GGC CCC TGG GCG GGA CCT GCC CTC     3264
Glu Glu Arg Thr Ala Cys Val Trp Gly Pro Trp Ala Gly Pro Ala Leu
        1075                1080                1085

ACT TTT GAT GAC TGC TGC TGC CGC CAG CCG CGG CTG GGT ACC CAG TGC     3312
Thr Phe Asp Asp Cys Cys Cys Arg Gln Pro Arg Leu Gly Thr Gln Cys
    1090                1095                1100

AGA CCG TGC CCG CCA CGT GGC ACC GGG TCC CAG TGC CCG ACT TCA CAG     3360
Arg Pro Cys Pro Pro Arg Gly Thr Gly Ser Gln Cys Pro Thr Ser Gln
1105                1110                1115                1120
```

```
AGT GAG AGC AAT TCT TTC TGG GAC ACA AGC CCC CTG CTA CTG GGG AAG      3408
Ser Glu Ser Asn Ser Phe Trp Asp Thr Ser Pro Leu Leu Leu Gly Lys
                1125                1130                1135

TCT CCG CGA GAC GAA GAC AGC TCA GAG GAG GAT TCA GAT GAG TGC CGT      3456
Ser Pro Arg Asp Glu Asp Ser Ser Glu Glu Asp Ser Asp Glu Cys Arg
            1140                1145                1150

TGT GTG AGC GGA CCG TGT GTG CCA CGG CCA GGC GGG GCG GTA TGC GAG      3504
Cys Val Ser Gly Pro Cys Val Pro Arg Pro Gly Gly Ala Val Cys Glu
        1155                1160                1165

TGT CCT GGA GGC TTT CAG CTG GAC GCC TCC CGT GCC CGC TGC GTG GAC      3552
Cys Pro Gly Gly Phe Gln Leu Asp Ala Ser Arg Ala Arg Cys Val Asp
    1170                1175                1180

ATT GAT GAG TGC CGA GAA CTG AAC CAG CGG GGA CTG CTG TGT AAG AGC      3600
Ile Asp Glu Cys Arg Glu Leu Asn Gln Arg Gly Leu Leu Cys Lys Ser
1185                1190                1195                1200

GAG CGG TGC GTG AAC ACC AGT GGA TCC TTC CGC TGT GTC TGC AAA GCT      3648
Glu Arg Cys Val Asn Thr Ser Gly Ser Phe Arg Cys Val Cys Lys Ala
                1205                1210                1215
```

```
GGC TTC ACG CGC AGC CGC CCT CAC GGG CCT GCG TGC CTC AGC GCC GCC     3696
Gly Phe Thr Arg Ser Arg Pro His Gly Pro Ala Cys Leu Ser Ala Ala
            1220                1225                1230

GCT GAT GAT GCA GCC ATA GCC CAC ACC TCA GTG ATC GAT CAT CGA GGG     3744
Ala Asp Asp Ala Ala Ile Ala His Thr Ser Val Ile Asp His Arg Gly
        1235                1240                1245

TAT TTT CAC                                                         3753
Tyr Phe His
    1250
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1251 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Met Arg Gln Ala Ala Leu Gly Leu Leu Ala Leu Leu Leu Leu Ala Leu
 1               5                  10                  15

Leu Gly Pro Gly Gly Arg Gly Val Gly Arg Pro Gly Ser Gly Ala Gln
            20                  25                  30

Ala Gly Ala Gly Arg Trp Ala Gln Arg Phe Lys Val Val Phe Ala Pro
        35                  40                  45

Val Ile Cys Lys Arg Thr Cys Leu Lys Gly Gln Cys Arg Asp Ser Cys
    50                  55                  60

Gln Gln Gly Ser Asn Met Thr Leu Ile Gly Glu Asn Gly His Ser Thr
65                  70                  75                  80

Asp Thr Leu Thr Gly Ser Ala Phe Arg Val Val Val Cys Pro Leu Pro
                85                  90                  95

Cys Met Asn Gly Gly Gln Cys Ser Ser Arg Asn Gln Cys Leu Cys Pro
            100                 105                 110

Pro Asp Phe Thr Gly Arg Phe Cys Gln Val Pro Ala Ala Gly Thr Gly
        115                 120                 125
```

```
Ala Gly Thr Gly Ser Ser Gly Pro Gly Trp Pro Asp Arg Ala Met Ser
    130                 135                 140

Thr Gly Pro Leu Pro Pro Leu Ala Pro Glu Gly Glu Ser Val Ala Ser
145                 150                 155                 160

Lys His Ala Ile Tyr Ala Val Gln Val Ile Ala Asp Pro Pro Gly Pro
                165                 170                 175

Gly Glu Gly Pro Pro Ala Gln His Ala Ala Phe Leu Val Pro Leu Gly
            180                 185                 190

Pro Gly Gln Ile Ser Ala Glu Val Gln Ala Pro Pro Pro Val Val Asn
        195                 200                 205

Val Arg Val His His Pro Pro Glu Ala Ser Val Gln Val His Arg Ile
    210                 215                 220

Glu Gly Pro Asn Ala Glu Gly Pro Ala Ser Ser Gln His Leu Leu Pro
225                 230                 235                 240

His Pro Lys Pro Pro His Pro Arg Pro Pro Thr Gln Lys Pro Leu Gly
                245                 250                 255
```

```
Arg Cys Phe Gln Asp Thr Leu Pro Lys Gln Pro Cys Gly Ser Asn Pro
            260                 265                 270

Leu Pro Gly Leu Thr Lys Gln Glu Asp Cys Cys Gly Ser Ile Gly Thr
        275                 280                 285

Ala Trp Gly Gln Ser Lys Cys His Lys Cys Pro Gln Leu Gln Tyr Thr
    290                 295                 300

Gly Val Gln Lys Pro Val Pro Val Arg Gly Glu Val Gly Ala Asp Cys
305                 310                 315                 320

Pro Gln Gly Tyr Lys Arg Leu Asn Ser Thr His Cys Gln Asp Ile Asn
                325                 330                 335

Glu Cys Ala Met Pro Gly Asn Val Cys His Gly Asp Cys Leu Asn Asn
            340                 345                 350

Pro Gly Ser Tyr Arg Cys Val Cys Pro Pro Gly His Ser Leu Gly Pro
        355                 360                 365

Leu Ala Ala Gln Cys Ile Ala Asp Lys Pro Glu Glu Lys Ser Leu Cys
    370                 375                 380
```

```
Phe Arg Leu Val Ser Thr Glu His Gln Cys Gln His Pro Leu Thr Thr
385                 390                 395                 400

Arg Leu Thr Arg Gln Leu Cys Cys Cys Ser Val Gly Lys Ala Trp Gly
                405                 410                 415

Ala Arg Cys Gln Arg Cys Pro Ala Asp Gly Thr Ala Ala Phe Lys Glu
            420                 425                 430

Ile Cys Pro Gly Trp Glu Arg Val Pro Tyr Pro His Leu Pro Pro Asp
        435                 440                 445

Ala His His Pro Gly Gly Lys Arg Leu Leu Pro Leu Pro Ala Pro Asp
    450                 455                 460

Gly Pro Pro Lys Pro Gln Gln Leu Pro Glu Ser Pro Ser Arg Ala Pro
465                 470                 475                 480

Pro Leu Glu Asp Thr Glu Glu Glu Arg Gly Val Thr Met Asp Pro Pro
                485                 490                 495

Val Ser Glu Glu Arg Ser Val Gln Gln Ser His Pro Thr Thr Thr Thr
            500                 505                 510
```

```
Ser Pro Pro Arg Pro Tyr Pro Glu Leu Ile Ser Arg Pro Ser Pro Pro
            515                 520                 525

Thr Phe His Arg Phe Leu Pro Asp Leu Pro Pro Ser Arg Ser Ala Val
        530                 535                 540

Glu Ile Ala Pro Thr Gln Val Thr Glu Thr Asp Glu Cys Arg Leu Asn
545                 550                 555                 560

Gln Asn Ile Cys Gly His Gly Gln Cys Val Pro Gly Pro Ser Asp Tyr
                565                 570                 575

Ser Cys His Cys Asn Ala Gly Tyr Arg Ser His Pro Gln His Arg Tyr
            580                 585                 590

Cys Val Asp Val Asn Glu Cys Glu Ala Glu Pro Cys Gly Pro Gly Lys
        595                 600                 605

Gly Ile Cys Met Asn Thr Gly Gly Ser Tyr Asn Cys His Cys Asn Arg
    610                 615                 620

Gly Tyr Arg Leu His Val Gly Ala Gly Gly Arg Ser Cys Val Asp Leu
625                 630                 635                 640
```

```
Asn Glu Cys Ala Lys Pro His Leu Cys Gly Asp Gly Gly Phe Cys Ile
                645                 650                 655

Asn Phe Pro Gly His Tyr Lys Cys Asn Cys Tyr Pro Gly Tyr Arg Leu
            660                 665                 670

Lys Ala Ser Arg Pro Pro Ile Cys Glu Asp Ile Asp Glu Cys Arg Asp
        675                 680                 685

Pro Ser Thr Cys Pro Asp Gly Lys Cys Glu Asn Lys Pro Gly Ser Phe
    690                 695                 700

Lys Cys Ile Ala Cys Gln Pro Gly Tyr Arg Ser Gln Gly Gly Gly Ala
705                 710                 715                 720

Cys Arg Asp Val Asn Glu Cys Ser Glu Gly Thr Pro Cys Ser Pro Gly
                725                 730                 735

Trp Cys Glu Lys Leu Pro Gly Ser Tyr Arg Cys Thr Cys Ala Gln Gly
            740                 745                 750

Ile Arg Thr Arg Thr Gly Arg Leu Ser Cys Ile Asp Val Asp Asp Cys
        755                 760                 765
```

```
Glu Ala Gly Lys Val Cys Gln Asp Gly Ile Cys Thr Asn Thr Pro Gly
    770                 775                 780

Ser Phe Gln Cys Gln Cys Leu Ser Gly Tyr His Leu Ser Arg Asp Arg
785                 790                 795                 800

Ser Arg Cys Glu Asp Ile Asp Glu Cys Asp Phe Pro Ala Ala Cys Ile
                805                 810                 815

Gly Gly Asp Cys Ile Asn Thr Asn Gly Ser Tyr Arg Cys Leu Cys Pro
            820                 825                 830

Leu Gly His Arg Leu Val Gly Gly Arg Lys Cys Lys Lys Asp Ile Asp
        835                 840                 845

Glu Cys Ser Gln Asp Pro Gly Leu Cys Leu Pro His Ala Cys Glu Asn
    850                 855                 860

Leu Gln Gly Ser Tyr Val Cys Val Cys Asp Glu Gly Phe Thr Leu Thr
865                 870                 875                 880

Gln Asp Gln His Gly Cys Glu Glu Val Glu Gln Pro His His Lys Lys
                885                 890                 895
```

```
Glu Cys Tyr Leu Asn Phe Asp Asp Thr Val Phe Cys Asp Ser Val Leu
            900                 905                 910

Ala Thr Asn Val Thr Gln Gln Glu Cys Cys Cys Ser Leu Gly Ala Gly
        915                 920                 925

Trp Gly Asp His Cys Glu Ile Tyr Pro Cys Pro Val Tyr Ser Ser Ala
    930                 935                 940

Glu Phe His Ser Leu Val Pro Asp Gly Lys Arg Leu His Ser Gly Gln
945                 950                 955                 960

Gln His Cys Glu Leu Cys Ile Pro Ala His Arg Asp Ile Asp Glu Cys
                965                 970                 975

Ile Leu Phe Gly Ala Glu Ile Cys Lys Glu Gly Lys Cys Val Asn Ser
            980                 985                 990

Gln Pro Gly Tyr Glu Cys Tyr Cys Lys Gln Gly Phe Tyr Tyr Asp Gly
        995                 1000                1005

Asn Leu Leu Glu Cys Val Asp Val Asp Glu Cys Leu Asp Glu Ser Asn
    1010                1015                1020
```

Cys Arg Asn Gly Val Cys Glu Asn Thr Trp Arg Leu Pro Cys Ala Cys
1025 1030 1035 1040

Thr Pro Pro Ala Glu Tyr Ser Pro Ala Gln Ala Gln Cys Leu Ile Pro
1045 1050 1055

Glu Arg Trp Ser Thr Pro Gln Arg Asp Val Lys Cys Ala Gly Ala Ser
1060 1065 1070

Glu Glu Arg Thr Ala Cys Val Trp Gly Pro Trp Ala Gly Pro Ala Leu
1075 1080 1085

Thr Phe Asp Asp Cys Cys Cys Arg Gln Pro Arg Leu Gly Thr Gln Cys
1090 1095 1100

Arg Pro [illegible] Pro Pro Arg Gly Thr Gly Ser Gln Cys Pro Thr Ser Gln
1105 1110 1115 1120

Ser Glu Ser Asn Ser Phe Trp Asp Thr Ser Pro Leu Leu Leu Gly Lys
1125 1130 1135

Ser Pro Arg Asp Glu Asp Ser Ser Glu Glu Asp Ser Asp Glu Cys Arg
1140 1145 1150

```
Cys Val Ser Gly Pro Cys Val Pro Arg Pro Gly Gly Ala Val Cys Glu
        1155                1160                1165

Cys Pro Gly Gly Phe Gln Leu Asp Ala Ser Arg Ala Arg Cys Val Asp
    1170                1175                1180

Ile Asp Glu Cys Arg Glu Leu Asn Gln Arg Gly Leu Leu Cys Lys Ser
1185                1190                1195                1200

Glu Arg Cys Val Asn Thr Ser Gly Ser Phe Arg Cys Val Cys Lys Ala
                1205                1210                1215

Gly Phe Thr Arg Ser Arg Pro His Gly Pro Ala Cys Leu Ser Ala Ala
            1220                1225                1230

Ala Asp Asp Ala Ala Ile Ala His Thr Ser Val Ile Asp His Arg Gly
        1235                1240                1245

Tyr Phe His
    1250
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AACATGACGC TCATCGGAGA GAAC 24

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AGGTGATCGC AGATCCTC 18

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TACCGATGCT ACCGCAGCAA TCTT 24

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATGCCTAAAC TCTACCAGCA CG 22

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GAGTCACGTC ATCCATTCCA CA 22

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CGTCCAAGTT GTGTCTTAGC AG 22

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Gly Pro Pro Gly Pro Gln Gly Ala Thr Gly Pro Leu Gly Pro Lys Gly
1               5                   10                  15

Gln Thr Gly Glu Pro Gly Ile Ala Gly Phe Lys Gly Glu Gln Gly Pro
            20                  25                  30

Lys Gly Glu Thr Gly Pro Ala Gly Pro Gln Gly Ala Pro Gly Pro Ala
        35                  40                  45

Gly Glu Glu Gly Lys
    50
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 159 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GGCCCTCCCG GTCCTCAAGG TGCAACTGGT CCTCTGGGCC CCAAAGGTCA GACGGGTGAG 60

CCCGGCATCG CTGGCTTCAA AGGTGAACAA GGCCCCAAGG GAGAGACTGG ACCTGCTGGG 120

CCCCAGGGAG CCCCTGGCCC TGCTGGTGAA GAAGGAAAA 159

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1442 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
Met Ile Arg Leu Gly Ala Pro Gln Ser Leu Val Leu Leu Thr Leu Leu
1               5                   10                  15

Ile Ala Ala Val Leu Arg Cys Gln Gly Gln Asp Ala Gln Glu Ala Gly
            20                  25                  30

Ser Cys Leu Gln Asn Gly Gln Arg Tyr Lys Asp Lys Asp Val Trp Lys
        35                  40                  45

Pro Ser Ser Cys Arg Ile Cys Val Cys Asp Thr Gly Asn Val Leu Cys
    50                  55                  60

Asp Asp Ile Ile Cys Glu Asp Pro Asp Cys Leu Asn Pro Glu Ile Pro
65                  70                  75                  80

Phe Gly Glu Cys Cys Pro Ile Cys Pro Ala Asp Leu Ala Thr Ala Ser
                85                  90                  95

Gly Arg Lys Leu Gly Pro Lys Gly Gln Lys Gly Glu Pro Gly Asp Ile
            100                 105                 110

Arg Asp Gly Pro Ala Gly Glu Gln Gly Pro Arg Gly Asp Arg Gly Asp
        115                 120                 125
```

```
Lys Gly Glu Lys Asn Phe Ala Ala Gln Met Ala Gly Gly Tyr Asp Glu
    130                 135                 140

Lys Ala Gly Gly Ala Gln Met Gly Val Met Gln Gly Pro Met Gly Pro
145                 150                 155                 160

Met Gly Pro Arg Gly Pro Pro Gly Pro Ala Gly Ala Pro Gly Pro Gln
                165                 170                 175

Gly Phe Gln Gly Asn Pro Gly Glu Pro Gly Glu Pro Gly Val Ser Gly
            180                 185                 190

Pro Met Gly Pro Arg Gly Pro Pro Gly Pro Ala Gly Lys Pro Gly Asp
        195                 200                 205

Asp Gly Glu Ala Gly Lys Pro Gly Lys Ser Gly Glu Arg Gly Leu Pro
    210                 215                 220

Gly Pro Met Gly Ala Arg Gly Phe Pro Gly Thr Pro Gly Leu Pro Gly
225                 230                 235                 240

Val Lys Gly His Arg Gly Tyr Pro Gly Leu Asp Gly Ala Lys Gly Glu
                245                 250                 255
```

```
Ala Gly Ala Pro Gly Val Lys Gly Glu Ser Gly Ser Pro Gly Glu Asn
            260                 265                 270

Gly Ser Pro Gly Pro Met Gly Pro Arg Gly Leu Pro Gly Glu Arg Gly
        275                 280                 285

Arg Thr Gly Pro Ala Gly Ala Ala Gly Ala Arg Gly Asn Asp Gly Gln
    290                 295                 300

Pro Gly Pro Ala Gly Pro Pro Gly Pro Val Gly Pro Ala Gly Gly Pro
305                 310                 315                 320

Gly Phe Pro Gly Ala Pro Gly Ala Lys Gly Glu Ala Gly Pro Thr Gly
                325                 330                 335

Ala Arg Gly Pro Glu Gly Ala Gln Gly Ser Arg Gly Glu Pro Gly Asn
            340                 345                 350

Pro Gly Ser Pro Gly Pro Ala Gly Ala Ser Gly Asn Pro Gly Thr Asp
        355                 360                 365

Gly Ile Pro Gly Ala Lys Gly Ser Ala Gly Ala Pro Gly Ile Ala Gly
    370                 375                 380
```

```
Ala Pro Gly Phe Pro Gly Pro Arg Gly Pro Pro Gly Pro Gln Gly Ala
385                 390                 395                 400

Thr Gly Pro Leu Gly Pro Lys Gly Gln Ala Gly Glu Pro Gly Ile Ala
                405                 410                 415

Gly Phe Lys Gly Asp Gln Gly Pro Lys Gly Glu Thr Gly Pro Ala Gly
            420                 425                 430

Pro Gln Gly Ala Pro Gly Pro Ala Gly Glu Glu Gly Lys Arg Gly Ala
        435                 440                 445

Arg Gly Glu Pro Gly Gly Ala Gly Pro Ile Gly Pro Pro Gly Glu Arg
    450                 455                 460

Gly Ala Pro Gly Asn Arg Gly Phe Pro Gly Gln Asp Gly Leu Ala Gly
465                 470                 475                 480

Pro Lys Gly Ala Pro Gly Glu Arg Gly Pro Ser Gly Leu Ala Gly Pro
                485                 490                 495

Lys Gly Ala Asn Gly Asp Pro Gly Arg Pro Gly Glu Pro Gly Leu Pro
            500                 505                 510
```

```
Gly Ala Arg Gly Leu Thr Gly Arg Pro Gly Asp Ala Gly Pro Gln Gly
        515                 520                 525

Lys Val Gly Pro Ser Gly Ala Pro Gly Glu Asp Gly Arg Pro Gly Pro
    530                 535                 540

Pro Gly Pro Gln Gly Ala Arg Gly Gln Pro Gly Val Met Gly Phe Pro
545                 550                 555                 560

Gly Pro Lys Gly Ala Asn Gly Glu Pro Gly Lys Ala Gly Glu Lys Gly
                565                 570                 575

Leu Ala Gly Ala Pro Gly Leu Arg Gly Leu Pro Gly Lys Asp Gly Glu
            580                 585                 590

Thr Gly Ala Ala Gly Pro Pro Gly Pro Ser Gly Pro Ala Gly Glu Arg
        595                 600                 605

Gly Glu Gln Gly Ala Pro Gly Pro Ser Gly Phe Gln Gly Leu Pro Gly
    610                 615                 620

Pro Pro Gly Pro Pro Gly Glu Gly Gly Lys Gln Gly Asp Gln Gly Ile
625                 630                 635                 640
```

Pro Gly Glu Ala Gly Ala Pro Gly Leu Val Gly Pro Arg Gly Glu Arg
645 650 655

Gly Phe Pro Gly Glu Arg Gly Ser Pro Gly Ala Gln Gly Leu Gln Gly
660 665 670

Pro Arg Gly Leu Pro Gly Thr Pro Gly Thr Asp Gly Pro Lys Gly Ala
675 680 685

Ala Gly Pro Asp Gly Pro Pro Gly Ala Gln Gly Pro Pro Gly Leu Gln
690 695 700

Gly Met Pro Gly Glu Arg Gly Ala Ala Gly Ile Ala Gly Pro Lys Gly
705 710 715 720

Asp Arg Gly Asp Val Gly Glu Lys Gly Pro Glu Gly Ala Pro Gly Lys
725 730 735

Asp Gly Gly Arg Gly Leu Thr Gly Pro Ile Gly Pro Pro Gly Pro Ala
740 745 750

Gly Ala Asn Gly Glu Lys Gly Glu Ala Gly Pro Pro Gly Pro Ser Gly
755 760 765

```
Ser Thr Gly Ala Arg Gly Ala Pro Gly Glu Pro Gly Glu Thr Gly Pro
    770                 775                 780

Pro Gly Pro Ala Gly Phe Ala Gly Pro Pro Gly Ala Asp Gly Gln Pro
785                 790                 795                 800

Gly Ala Lys Gly Asp Gln Gly Glu Ala Gly Gln Lys Gly Asp Ala Gly
                805                 810                 815

Ala Pro Gly Pro Gln Gly Pro Ser Gly Ala Pro Gly Pro Gln Gly Pro
            820                 825                 830

Thr Gly Val Thr Gly Pro Lys Gly Ala Arg Gly Ala Gln Gly Pro Pro
        835                 840                 845

Gly Ala Thr Gly Phe Pro Gly Ala Ala Gly Arg Val Gly Pro Pro Gly
    850                 855                 860

Ala Asn Gly Asn Pro Gly Pro Ala Gly Pro Pro Gly Pro Ala Gly Lys
865                 870                 875                 880

Asp Gly Pro Lys Gly Val Arg Gly Asp Ser Gly Pro Pro Gly Arg Ala
                885                 890                 895
```

```
Gly Asp Pro Gly Leu Glu Gly Pro Ala Gly Ala Pro Gly Glu Lys Gly
                900                     905                     910

Glu Pro Gly Asp Asp Gly Pro Ser Gly Leu Asp Gly Pro Pro Gly Pro
            915                     920                     925

Gln Gly Leu Ala Gly Gln Arg Gly Ile Val Gly Leu Pro Gly Gln Arg
    930                     935                     940

Gly Glu Arg Gly Phe Pro Gly Leu Pro Gly Pro Ser Gly Glu Pro Gly
945                     950                     955                     960

Lys Gln Gly Ala Pro Gly Ala Ser Gly Asp Arg Gly Pro Pro Gly Pro
                    965                     970                     975

Val Gly Pro Pro Gly Leu Thr Gly Pro Ala Gly Glu Pro Gly Arg Glu
                980                     985                     990

Gly Ser Pro Gly Ala Asp Gly Pro Pro Gly Arg Asp Gly Ala Ala Gly
            995                     1000                    1005

Val Lys Gly Asp Arg Gly Glu Thr Gly Ala Leu Gly Ala Pro Gly Ala
    1010                    1015                    1020
```

```
Pro Gly Pro Pro Gly Ser Pro Gly Pro Ala Gly Pro Thr Gly Lys Gln
1025                1030                1035                1040

Gly Asp Arg Gly Glu Ala Gly Ala Gln Gly Pro Met Gly Pro Ser Gly
                1045                1050                1055

Pro Ala Gly Ala Arg Gly Ile Ala Gly Pro Gln Gly Pro Arg Gly Asp
            1060                1065                1070

Lys Gly Glu Ser Gly Glu Gln Gly Glu Arg Gly Leu Lys Gly His Arg
        1075                1080                1085

Gly Phe Thr Gly Leu Gln Gly Leu Pro Gly Pro Pro Gly Pro Ser Gly
    1090                1095                1100

Asp Gln Gly Ala Ser Gly Pro Ala Gly Pro Ser Gly Pro Arg Gly Pro
1105                1110                1115                1120

Pro Gly Pro Val Gly Pro Ser Gly Lys Asp Gly Ser Asn Gly Ile Pro
                1125                1130                1135

Gly Pro Ile Gly Pro Pro Gly Pro Arg Gly Arg Ser Gly Glu Thr Gly
            1140                1145                1150
```

Pro Val Gly Pro Pro Gly Ser Pro Gly Pro Pro Gly Pro Pro Gly Pro
1155 1160 1165

Pro Gly Pro Gly Ile Asp Met Ser Ala Phe Ala Gly Leu Gly Gln Arg
1170 1175 1180

Glu Lys Gly Pro Asp Pro Met Gln Tyr Met Arg Ala Asp Glu Ala Asp
1185 1190 1195 1200

Ser Thr Leu Arg Gln His Asp Val Glu Val Asp Ala Thr Leu Lys Ser
1205 1210 1215

Leu Asn Asn Gln Ile Glu Ser Ile Arg Ser Pro Asp Gly Ser Arg Lys
1220 1225 1230

Asn Pro Ala Arg Thr Cys Gln Asp Leu Lys Leu Cys His Pro Glu Trp
1235 1240 1245

Lys Ser Gly Asp Tyr Trp Ile Asp Pro Asn Gln Gly Cys Thr Leu Asp
1250 1255 1260

Ala Met Lys Val Phe Cys Asn Met Glu Thr Gly Glu Thr Cys Val Tyr
1265 1270 1275 1280

```
Pro Asn Pro Ala Thr Val Pro Arg Lys Asn Trp Trp Ser Ser Lys Ser
                1285                1290                1295

Lys Glu Lys Lys His Ile Trp Phe Gly Glu Thr Met Asn Gly Gly Phe
            1300                1305                1310

His Phe Ser Tyr Gly Asp Gly Asn Leu Ala Pro Asn Thr Ala Asn Val
        1315                1320                1325

Gln Met Thr Phe Leu Arg Leu Leu Ser Thr Glu Gly Ser Gln Asn Ile
    1330                1335                1340

Thr Tyr His Cys Lys Asn Ser Ile Ala Tyr Leu Asp Glu Ala Ala Gly
1345                1350                1355                1360

Asn Leu Lys Lys Ala Leu Leu Ile Gln Gly Ser Asn Asp Val Glu Met
                1365                1370                1375

Arg Ala Glu Gly Asn Ser Arg Phe Thr Tyr Thr Ala Leu Lys Asp Gly
            1380                1385                1390

Cys Thr Lys His Thr Gly Lys Trp Gly Lys Thr Val Ile Glu Tyr Arg
        1395                1400                1405
```

Ser Gln Lys Thr Ser Arg Leu Pro Ile Ile Asp Ile Ala Pro Met Asp
    1410   1415   1420

Ile Gly Gly Ala Glu Gln Glu Phe Gly Val Asp Ile Gly Pro Val Cys
1425   1430   1435   1440

Phe Leu

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 267 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ATAGGCCCTT TGGAGACGGC TGTTTTCCAG ACTCCAAACT ATCGTGTCAC ACGTGTGGGA 60

AATGAAGTGT CTTTCAATTG TGAGCAAACC CTGGACCACA ATACTATGTA CTGGTACAAG 120

CAAGACTCTA AGAAATTGCT GAAGATTATG TTTAGCTACA ATAATAAGCA ACTCATTGTA 180

AACGAAACAG TTCCAAGGCG CTTCTCACCT CAGTCTTCAG ATAAAGCTCA TTTGAATCTT 240

CGAATCAAGT CTGTAGAGCT GGAGGAC 267

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
Gly Pro Ser Gly Asp Gln Gly Ala Ser Gly Pro Ala Gly Pro Ser Gly
1               5                   10                  15

Pro Arg Gly Pro Pro Gly Pro Val Gly Pro Ser Gly Lys Asp Gly Ala
            20                  25                  30

Asn Gly Ile Pro Gly Pro Ile Gly Pro Pro Gly Pro Arg Gly Arg Ser
        35                  40                  45

Gly Glu Thr Gly Pro Ala
    50
```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 731 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
AGAATATAGA TAGATATGTC TGTGCTGACC GTGGCCTTTT GCCTCTTCCT TCTACACAGG    60
GTCCTTCTGG AGACCAAGGT GCTTCTGGTC CTGCTGGTCC TTCTGGCCCT AGAGTAAGTG   120
ACATGGAGTT GGAAGATGGA GGGGGCCCTT CAGAGAGTGT GGGCCTGTGT TCCCATGGGG   180
AGGGAAATGC TGCTGCTTCT GGGGAAGCTG TGGGCTCAGG GGTCCTCACT CAGTAATGGG   240
GGCAGGACTG GCTCATGTGC CTATGGCCAG AAAAGCGCCT GAGGCCACAA TGGCTGTAAG   300
ACAAACATGA ATCAGCCTCT CGCTGTCAGA CAGAACAGCA TTTTACAAAG AGGAGCTTAG   360
GAGGGTAGGC AAGCCATGGA GCTATCCTGC TGGTTCTTGG CCAAATAGAG ACCAACTTAG   420
GGTTCCATGA CTGAGCATGT GAAGAACTGG GGGCGGAGTG GCTGGTGCTA TCAGGACAGC   480
CACCTACCCA GCCCCAGCGA CTCCCCAGCC TTCCCTGTGG TGACCACTCT TTCCTCACGA   540
CCTCTCTCTC TTGCAGGGTC CTCCTGGCCC CGTCGGTCCC TCTGGCAAAG ATGGTGCTAA   600
TGGAATCCCT GGCCCCATTG GGCCTCCTGG TCCCCGTGGA CGATCAGGCG AAACCGGCCC   660
```

TGCTGTAAGT GTCCTGACTC CTTCCCTGCT GTCGAGGTGT CCCTACCATC CGGGAGGCTT 720

GAGCTCTTTT T 731

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```
Gly Glu Ser Val Ala Ser Lys His Ala Ile Tyr Ala Val Cys
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 5502 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
    (A) DESCRIPTION: /desc = "DNA"

(ix) FEATURE:
    (A) NAME/KEY: CDS
    (B) LOCATION: 1..5502

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

```
ATG GAG AGC ACC TCC CCG CGA GGT CTC CGG TGC CCA CAG CTC TGC AGC      48
Met Glu Ser Thr Ser Pro Arg Gly Leu Arg Cys Pro Gln Leu Cys Ser
            1255                1260                1265
```

```
CAC TCT GGC GCC ATG AGA GCG CCG ACC ACC GCT CGC TGC TCC GGA TGC       96
His Ser Gly Ala Met Arg Ala Pro Thr Thr Ala Arg Cys Ser Gly Cys
        1270                1275                1280

ATC CAA CGG GTG CGT TGG AGG GGC TTC CTG CCA CTT GTC CTG GCT GTC      144
Ile Gln Arg Val Arg Trp Arg Gly Phe Leu Pro Leu Val Leu Ala Val
    1285                1290                1295

TTG ATG GGG ACA AGT CAT GCC CAA CGG GAT TCC ATA GGG AGA TAC GAA      192
Leu Met Gly Thr Ser His Ala Gln Arg Asp Ser Ile Gly Arg Tyr Glu
1300                1305                1310                1315

CCA GCT AGC AGG GAT GCG AAT CGG TTG TGG CAC CCC GTG GGC AGC CAC      240
Pro Ala Ser Arg Asp Ala Asn Arg Leu Trp His Pro Val Gly Ser His
                1320                1325                1330

CCC GCA GCG GCT GCA GCC AAG GTG TAC AGT CTG TTC CGA GAG CCT GAC      288
Pro Ala Ala Ala Ala Ala Lys Val Tyr Ser Leu Phe Arg Glu Pro Asp
            1335                1340                1345

GCG CCG GTC CCC GGC TTG TCG CCC TCT GAG TGG AAC CAG CCG GCC CAG      336
Ala Pro Val Pro Gly Leu Ser Pro Ser Glu Trp Asn Gln Pro Ala Gln
        1350                1355                1360
```

```
GGG AAC CCG GGA TGG CTC GCA GAG GCC GAG GCC AGG AGG CCA CCT CGA      384
Gly Asn Pro Gly Trp Leu Ala Glu Ala Glu Ala Arg Arg Pro Pro Arg
    1365                1370                1375

ACC CAG CAG CTG CGT CGA GTC CAG CCA CCT GTC CAG ACT CGG AGA AGC      432
Thr Gln Gln Leu Arg Arg Val Gln Pro Pro Val Gln Thr Arg Arg Ser
1380                1385                1390                1395

CAT CCC CGG GGC CAG CAG CAG ATA GCA GCC CGG GCT GCA CCT TCT GTC      480
His Pro Arg Gly Gln Gln Gln Ile Ala Ala Arg Ala Ala Pro Ser Val
                1400                1405                1410

GCG CGC CTG GAA ACC CCT CAG CGA CCC GCG GCT GCA CGG CGA GGG CGG      528
Ala Arg Leu Glu Thr Pro Gln Arg Pro Ala Ala Ala Arg Arg Gly Arg
            1415                1420                1425

CTC ACT GGG AGA AAT GTC TGC GGG GGA CAG TGC TGC CCA GGA TGG ACA      576
Leu Thr Gly Arg Asn Val Cys Gly Gly Gln Cys Cys Pro Gly Trp Thr
        1430                1435                1440

ACA TCA AAC AGC ACC AAC CAC TGT ATC AAA CCT GTG TGT CAG CCT CCC      624
Thr Ser Asn Ser Thr Asn His Cys Ile Lys Pro Val Cys Gln Pro Pro
    1445                1450                1455
```

```
TGT CAG AAC CGA GGC TCC TGC AGC AGG CCC CAG GTC TGC ATC TGC CGT      672
Cys Gln Asn Arg Gly Ser Cys Ser Arg Pro Gln Val Cys Ile Cys Arg
1460                1465                1470                1475

TCT GGC TTC CGT GGG GCG CGC TGT GAG GAG GTC ATC CCT GAG GAG GAA      720
Ser Gly Phe Arg Gly Ala Arg Cys Glu Glu Val Ile Pro Glu Glu Glu
                1480                1485                1490

TTT GAC CCT CAG AAT GCC AGG CCT GTG CCC AGA CGC TCA GTG GAG AGA      768
Phe Asp Pro Gln Asn Ala Arg Pro Val Pro Arg Arg Ser Val Glu Arg
            1495                1500                1505

GCA CCC GGT CCT CAC AGA AGC AGT GAG GCC AGA GGA AGT CTA GTG ACC      816
Ala Pro Gly Pro His Arg Ser Ser Glu Ala Arg Gly Ser Leu Val Thr
        1510                1515                1520

AGA ATA CAG CCG CTG GTA CCA CCA CCA TCA CCA CCT CCA TCT CGG CGC      864
Arg Ile Gln Pro Leu Val Pro Pro Pro Ser Pro Pro Pro Ser Arg Arg
    1525                1530                1535

CTC AGC CAG CCC TGG CCC CTG CAG CAG CAC TCA GGG CCG TCC AGG ACA      912
Leu Ser Gln Pro Trp Pro Leu Gln Gln His Ser Gly Pro Ser Arg Thr
1540                1545                1550                1555
```

```
GTT CGT CGG TAT CCG GCC ACT GGT GCC AAT GGC CAG CTG ATG TCC AAC      960
Val Arg Arg Tyr Pro Ala Thr Gly Ala Asn Gly Gln Leu Met Ser Asn
                1560                1565                1570

GCT TTG CCT TCA GGA CTC GAG CTG AGA GAC AGC AGC CCA CAG GCA GCA     1008
Ala Leu Pro Ser Gly Leu Glu Leu Arg Asp Ser Ser Pro Gln Ala Ala
            1575                1580                1585

CAT GTG AAC CAT CTC TCA CCC CCC TGG GGG CTG AAC CTC ACC GAG AAA     1056
His Val Asn His Leu Ser Pro Pro Trp Gly Leu Asn Leu Thr Glu Lys
        1590                1595                1600

ATC AAG AAA ATC AAA GTC GTC TTC ACC CCC ACC ATC TGC AAG CAG ACC     1104
Ile Lys Lys Ile Lys Val Val Phe Thr Pro Thr Ile Cys Lys Gln Thr
    1605                1610                1615

TGT GCC CGG GGA CGC TGT GCC AAC AGC TGT GAG AAG GGT GAC ACC ACC     1152
Cys Ala Arg Gly Arg Cys Ala Asn Ser Cys Glu Lys Gly Asp Thr Thr
1620                1625                1630                1635

ACC TTG TAC AGT CAG GGT GGC CAT GGG CAT GAC CCC AAG TCT GGC TTC     1200
Thr Leu Tyr Ser Gln Gly Gly His Gly His Asp Pro Lys Ser Gly Phe
                1640                1645                1650
```

```
CGT ATC TAT TTC TGC CAA ATC CCC TGC CTG AAT GGT GGC CGC TGC ATC     1248
Arg Ile Tyr Phe Cys Gln Ile Pro Cys Leu Asn Gly Gly Arg Cys Ile
                1655                1660                1665

GGC CGG GAC GAG TGC TGG TGT CCA GCC AAC TCC ACA GGA AAG TTC TGC     1296
Gly Arg Asp Glu Cys Trp Cys Pro Ala Asn Ser Thr Gly Lys Phe Cys
            1670                1675                1680

CAT CTG CCT GTC CCG CAG CCA GAC AGG GAA CCT GCA GGG CGA GGT TCC     1344
His Leu Pro Val Pro Gln Pro Asp Arg Glu Pro Ala Gly Arg Gly Ser
        1685                1690                1695

CGG CAC AGA ACC CTG CTG GAA GGT CCC CTG AAG CAA TCC ACC TTC ACG     1392
Arg His Arg Thr Leu Leu Glu Gly Pro Leu Lys Gln Ser Thr Phe Thr
1700                1705                1710                1715

CTG CCT CTC TCT AAC CAG CTC GCC TCT GTG AAC CCC TCG CTG GTG AAG     1440
Leu Pro Leu Ser Asn Gln Leu Ala Ser Val Asn Pro Ser Leu Val Lys
                1720                1725                1730

GTG CAA ATT CAT CAC CCG CCT GAG GCC TCT GTG CAG ATT CAC CAG GTG     1488
Val Gln Ile His His Pro Pro Glu Ala Ser Val Gln Ile His Gln Val
                1735                1740                1745
```

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GCC CGG GTC CGG GGT GAG CTG GAC CCC GTG CTG GAG GAC AAC AGT GTG      1536
Ala Arg Val Arg Gly Glu Leu Asp Pro Val Leu Glu Asp Asn Ser Val
        1750                1755                1760

GAG ACC AGA GCC TCT CAT CGC CCC CAC GGC AAC CTA GGC CAC AGC CCC      1584
Glu Thr Arg Ala Ser His Arg Pro His Gly Asn Leu Gly His Ser Pro
    1765                1770                1775

TGG GCC AGC AAC AGC ATA CCC GCT CGG GCC GGA GAG GCC CCT CGG CCA      1632
Trp Ala Ser Asn Ser Ile Pro Ala Arg Ala Gly Glu Ala Pro Arg Pro
1780                1785                1790                1795

CCA CCA GTG CTG TCT AGG CAT TAT GGA CTT CTG GGC CAG TGT TAC CTG      1680
Pro Pro Val Leu Ser Arg His Tyr Gly Leu Leu Gly Gln Cys Tyr Leu
                1800                1805                1810

AGC ACG GTG AAT GGA CAG TGT GCT AAC CCC CTA GGT AGT CTG ACT TCT      1728
Ser Thr Val Asn Gly Gln Cys Ala Asn Pro Leu Gly Ser Leu Thr Ser
            1815                1820                1825

CAG GAG GAC TGC TGT GGC AGT GTG GGG ACC TTC TGG GGG GTG ACC TCC      1776
Gln Glu Asp Cys Cys Gly Ser Val Gly Thr Phe Trp Gly Val Thr Ser
        1830                1835                1840
```

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TGT GCT CCC TGC CCA CCC AGA CAA GAG GGT CCA GCC TTC CCA GTG ATT    1824
Cys Ala Pro Cys Pro Pro Arg Gln Glu Gly Pro Ala Phe Pro Val Ile
    1845                1850                1855

GAA AAT GGC CAG CTG GAG TGT CCC CAA GGA TAC AAG AGA CTG AAC CTC    1872
Glu Asn Gly Gln Leu Glu Cys Pro Gln Gly Tyr Lys Arg Leu Asn Leu
1860                1865                1870                1875

AGC CAC TGC CAA GAT ATC AAT GAG TGC CTG ACC CTG GGC CTC TGC AAG    1920
Ser His Cys Gln Asp Ile Asn Glu Cys Leu Thr Leu Gly Leu Cys Lys
                1880                1885                1890

GAC TCG GAG TGC GTG AAC ACC AGG GGC AGC TAC CTG TGC ACC TGC AGG    1968
Asp Ser Glu Cys Val Asn Thr Arg Gly Ser Tyr Leu Cys Thr Cys Arg
            1895                1900                1905

CCT GGC CTC ATG CTG GAT CCG TCA AGG AGC CGC TGC GTA TCG GAC AAG    2016
Pro Gly Leu Met Leu Asp Pro Ser Arg Ser Arg Cys Val Ser Asp Lys
        1910                1915                1920

GCT GTC TCC ATG CAG CAG GGA CTA TGC TAC CGG TCA CTG GGG TCT GGT    2064
Ala Val Ser Met Gln Gln Gly Leu Cys Tyr Arg Ser Leu Gly Ser Gly
    1925                1930                1935
```

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ACC TGC ACC CTG CCT TTG GTT CAT CGG ATC ACC AAG CAG ATA TGC TGC      2112
Thr Cys Thr Leu Pro Leu Val His Arg Ile Thr Lys Gln Ile Cys Cys
1940                1945                1950                1955

TGC AGC CGT GTG GGC AAA GCC TGG GGT AGC ACA TGT GAA CAG TGT CCC      2160
Cys Ser Arg Val Gly Lys Ala Trp Gly Ser Thr Cys Glu Gln Cys Pro
                1960                1965                1970

CTG CCT GGC ACA GAA GCC TTC AGG GAG ATC TGC CCT GCT GGC CAT GGC      2208
Leu Pro Gly Thr Glu Ala Phe Arg Glu Ile Cys Pro Ala Gly His Gly
            1975                1980                1985

TAC ACC TAC TCG AGC TCA GAC ATC CGC CTG TCT ATG AGG AAA GCC GAA      2256
Tyr Thr Tyr Ser Ser Ser Asp Ile Arg Leu Ser Met Arg Lys Ala Glu
        1990                1995                2000

GAA GAG GAA CTG GCT AGC CCC TTA AGG GAG CAG ACA GAG CAG AGC ACT      2304
Glu Glu Glu Leu Ala Ser Pro Leu Arg Glu Gln Thr Glu Gln Ser Thr
    2005                2010                2015

GCA CCC CCA CCT GGG CAA GCA GAG AGG CAA CCA CTC CGG GCA GCC ACC      2352
Ala Pro Pro Pro Gly Gln Ala Glu Arg Gln Pro Leu Arg Ala Ala Thr
2020                2025                2030                2035
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GCC ACC TGG ATT GAG GCT GAG ACC CTC CCT GAC AAA GGT GAC TCT CGG      2400
Ala Thr Trp Ile Glu Ala Glu Thr Leu Pro Asp Lys Gly Asp Ser Arg
                2040                 2045                 2050

GCT GTT CAG ATC ACA ACC AGT GCT CCC CAC CTA CCT GCC CGG GTA CCA      2448
Ala Val Gln Ile Thr Thr Ser Ala Pro His Leu Pro Ala Arg Val Pro
            2055                 2060                 2065

GGG GAT GCC ACT GGA AGA CCA GCA CCA TCC TTG CCT GGA CAG GGC ATT      2496
Gly Asp Ala Thr Gly Arg Pro Ala Pro Ser Leu Pro Gly Gln Gly Ile
        2070                 2075                 2080

CCA GAG AGT CCA GCA GAA GAG CAA GTG ATT CCC TCC AGT GAT GTC TTG      2544
Pro Glu Ser Pro Ala Glu Glu Gln Val Ile Pro Ser Ser Asp Val Leu
    2085                 2090                 2095

GTG ACA CAC AGC CCC CCA GAC TTT GAT CCA TGT TTT GCT GGA GCC TCC      2592
Val Thr His Ser Pro Pro Asp Phe Asp Pro Cys Phe Ala Gly Ala Ser
2100                 2105                 2110                 2115

AAC ATC TGT GGC CCT GGG ACC TGT GTG AGC CTC CCA AAT GGA TAC AGA      2640
Asn Ile Cys Gly Pro Gly Thr Cys Val Ser Leu Pro Asn Gly Tyr Arg
                2120                 2125                 2130
```

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TGT GTC TGC AGC CCT GGC TAC CAG CTA CAC CCC AGC CAA GAC TAC TGT      2688
Cys Val Cys Ser Pro Gly Tyr Gln Leu His Pro Ser Gln Asp Tyr Cys
                2135                     2140                2145

ACT GAT GAC AAC GAG TGT ATG AGG AAC CCC TGT GAA GGA AGA GGG CGC      2736
Thr Asp Asp Asn Glu Cys Met Arg Asn Pro Cys Glu Gly Arg Gly Arg
            2150                2155                2160

TGT GTC AAC AGT GTG GGC TCC TAC TCC TGC CTC TGC TAT CCT GGC TAC      2784
Cys Val Asn Ser Val Gly Ser Tyr Ser Cys Leu Cys Tyr Pro Gly Tyr
    2165                2170                2175

ACA CTA GTC ACC CTC GGA GAC ACA CAG GAG TGC CAA GAT ATC GAT GAG      2832
Thr Leu Val Thr Leu Gly Asp Thr Gln Glu Cys Gln Asp Ile Asp Glu
2180                2185                2190                2195

TGT GAG CAG CCC GGG GTG TGC AGT GGT GGG CGA TGC AGC AAC ACG GAG      2880
Cys Glu Gln Pro Gly Val Cys Ser Gly Gly Arg Cys Ser Asn Thr Glu
                2200                2205                2210

GGC TCG TAC CAC TGC GAG TGT GAT CGG GGC TAC ATC ATG GTC AGG AAA      2928
Gly Ser Tyr His Cys Glu Cys Asp Arg Gly Tyr Ile Met Val Arg Lys
            2215                2220                2225
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GGA CAC TGT CAA GAT ATC AAC GAA TGC CGT CAC CCT GGT ACC TGC CCT      2976
Gly His Cys Gln Asp Ile Asn Glu Cys Arg His Pro Gly Thr Cys Pro
        2230                2235                2240

GAT GGG AGA TGC GTC AAC TCC CCT GGC TCC TAC ACT TGT CTG GCC TGT      3024
Asp Gly Arg Cys Val Asn Ser Pro Gly Ser Tyr Thr Cys Leu Ala Cys
    2245                2250                2255

GAG GAG GGC TAT GTA GGC CAG AGT GGG AGC TGT GTA GAT GTC AAT GAG      3072
Glu Glu Gly Tyr Val Gly Gln Ser Gly Ser Cys Val Asp Val Asn Glu
2260                2265                2270                2275

TGT CTG ACC CCT GGG ATA TGT ACC CAT GGA AGG TGC ATC AAC ATG GAA      3120
Cys Leu Thr Pro Gly Ile Cys Thr His Gly Arg Cys Ile Asn Met Glu
                2280                2285                2290

GGC TCC TTT AGA TGC TCC TGT GAG CCG GGC TAT GAG GTC ACC CCA GAC      3168
Gly Ser Phe Arg Cys Ser Cys Glu Pro Gly Tyr Glu Val Thr Pro Asp
            2295                2300                2305

AAG AAG GGC TGC CGA GAT GTG GAC GAG TGT GCC AGC CGA GCC TCG TGC      3216
Lys Lys Gly Cys Arg Asp Val Asp Glu Cys Ala Ser Arg Ala Ser Cys
        2310                2315                2320
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CCC ACG GGC CTC TGC CTC AAC ACG GAG GGC TCC TTC ACC TGC TCA GCC      3264
Pro Thr Gly Leu Cys Leu Asn Thr Glu Gly Ser Phe Thr Cys Ser Ala
    2325                    2330                    2335

TGT CAG AGC GGG TAC TGG GTG AAC GAA GAT GGC ACT GCC TGT GAA GAC      3312
Cys Gln Ser Gly Tyr Trp Val Asn Glu Asp Gly Thr Ala Cys Glu Asp
2340                    2345                    2350                2355

TTG GAT GAA TGT GCC TTC CCT GGA GTC TGC CCC ACA GGC GTC TGC ACC      3360
Leu Asp Glu Cys Ala Phe Pro Gly Val Cys Pro Thr Gly Val Cys Thr
                2360                    2365                2370

AAT ACT GTA GGC TCC TTC TCC TGC AAG GAC TGT GAC CAG GGC TAC CGG      3408
Asn Thr Val Gly Ser Phe Ser Cys Lys Asp Cys Asp Gln Gly Tyr Arg
            2375                    2380                2385

CCC AAC CCC CTG GGC AAC AGA TGC GAA GAT GTG GAT GAG TGT GAA GGT      3456
Pro Asn Pro Leu Gly Asn Arg Cys Glu Asp Val Asp Glu Cys Glu Gly
        2390                    2395                2400

CCC CAA AGC AGC TGC CGG GGA GGC GAA TGC AAG AAC ACA GAA GGT TCC      3504
Pro Gln Ser Ser Cys Arg Gly Gly Glu Cys Lys Asn Thr Glu Gly Ser
    2405                    2410                    2415
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TAC CAA TGC CTC TGT CAC CAG GGC TTC CAG CTG GTC AAT GGC ACC ATG    3552
Tyr Gln Cys Leu Cys His Gln Gly Phe Gln Leu Val Asn Gly Thr Met
2420                2425                2430                2435

TGT GAG GAC GTG AAT GAG TGT GTT GGG GAA GAG CAT TGT GCT CCT CAC    3600
Cys Glu Asp Val Asn Glu Cys Val Gly Glu Glu His Cys Ala Pro His
                2440                2445                2450

GGC GAG TGC CTC AAC AGC CTG GGC TCC TTC TTC TGC CTC TGT GCA CCC    3648
Gly Glu Cys Leu Asn Ser Leu Gly Ser Phe Phe Cys Leu Cys Ala Pro
            2455                2460                2465

GGC TTT GCT AGT GCT GAG GGG GGC ACC AGA TGC CAG GAT GTT GAT GAA    3696
Gly Phe Ala Ser Ala Glu Gly Gly Thr Arg Cys Gln Asp Val Asp Glu
        2470                2475                2480

TGT GCA GCC ACA GAC CCG TGT CCG GGA GGA CAC TGT GTC AAC ACA GAG    3744
Cys Ala Ala Thr Asp Pro Cys Pro Gly Gly His Cys Val Asn Thr Glu
    2485                2490                2495

GGC TCC TTC AGC TGT CTG TGT GAG ACT GCT TCC TTC CAG CCC TCC CCA    3792
Gly Ser Phe Ser Cys Leu Cys Glu Thr Ala Ser Phe Gln Pro Ser Pro
2500                2505                2510                2515
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GAC AGC GGA GAA TGT TTG GAT ATT GAT GAG TGT GAG GAC CGT GAA GAC     3840
Asp Ser Gly Glu Cys Leu Asp Ile Asp Glu Cys Glu Asp Arg Glu Asp
                2520                2525                2530

CCG GTG TGC GGA GCC TGG AGG TGT GAG AAC AGT CCT GGT TCC TAC CGC     3888
Pro Val Cys Gly Ala Trp Arg Cys Glu Asn Ser Pro Gly Ser Tyr Arg
            2535                2540                2545

TGC ATC CTG GAC TGC CAG CCT GGA TTC TAT GTG GCG CCA AAT GGA GAC     3936
Cys Ile Leu Asp Cys Gln Pro Gly Phe Tyr Val Ala Pro Asn Gly Asp
        2550                2555                2560

TGC ATT GAC ATA GAT GAA TGT GCC AAT GAC ACT GTG TGT GGG AAC CAT     3984
Cys Ile Asp Ile Asp Glu Cys Ala Asn Asp Thr Val Cys Gly Asn His
    2565                2570                2575

GGC TTC TGT GAC AAC ACG GAC GGC TCC TTC CGC TGC CTG TGT GAC CAG     4032
Gly Phe Cys Asp Asn Thr Asp Gly Ser Phe Arg Cys Leu Cys Asp Gln
2580                2585                2590                2595

GGC TTC GAG ACC TCA CCA TCA GGC TGG GAG TGT GTT GAT GTG AAC GAG     4080
Gly Phe Glu Thr Ser Pro Ser Gly Trp Glu Cys Val Asp Val Asn Glu
                2600                2605                2610
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TGT GAG CTC ATG ATG GCA GTG TGT GGG GAT GCG CTC TGT GAG AAC GTG      4128
Cys Glu Leu Met Met Ala Val Cys Gly Asp Ala Leu Cys Glu Asn Val
                2615                2620                2625

GAA GGC TCC TTC CTG TGC CTT TGC GCC AGT GAC CTT GAG GAG TAC GAC      4176
Glu Gly Ser Phe Leu Cys Leu Cys Ala Ser Asp Leu Glu Glu Tyr Asp
            2630                2635                2640

GCA GAA GAA GGA CAC TGC CGT CCT CGG GTG GCT GGA GCT CAG AGA ATC      4224
Ala Glu Glu Gly His Cys Arg Pro Arg Val Ala Gly Ala Gln Arg Ile
    2645                2650                2655

CCA GAG GTC CGG ACA GAG GAC CAG GCT CCA AGC CTT ATC CGC ATG GAA      4272
Pro Glu Val Arg Thr Glu Asp Gln Ala Pro Ser Leu Ile Arg Met Glu
2660                2665                2670                2675

TGC TAC TCT GAA CAC AAT GGT GGT CCT CCC TGC TCT CAA ATC CTG GGC      4320
Cys Tyr Ser Glu His Asn Gly Gly Pro Pro Cys Ser Gln Ile Leu Gly
                    2680                2685                2690

CAG AAC TCC ACA CAG GCC GAG TGC TGC TGC ACT CAG GGT GCC AGA TGG      4368
Gln Asn Ser Thr Gln Ala Glu Cys Cys Cys Thr Gln Gly Ala Arg Trp
                2695                2700                2705
```

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GGA AAG GCC TGT GCG CCC TGC CCA TCT GAG GAC TCA GTT GAA TTC AGT      4416
Gly Lys Ala Cys Ala Pro Cys Pro Ser Glu Asp Ser Val Glu Phe Ser
        2710                2715                2720

CAG CTC TGC CCC AGT GGT CAA GGT TAC ATC CCA GTG GAA GGA GCC TGG      4464
Gln Leu Cys Pro Ser Gly Gln Gly Tyr Ile Pro Val Glu Gly Ala Trp
    2725                2730                2735

ACA TTT GGA CAA ACC ATG TAT ACA GAT GCC GAT GAA TGT GTA CTG TTT      4512
Thr Phe Gly Gln Thr Met Tyr Thr Asp Ala Asp Glu Cys Val Leu Phe
2740                2745                2750                2755

GGG CCT GCT CTC TGC CAG AAT GGC CGA TGC TCA AAC ATA GTG CCT GGC      4560
Gly Pro Ala Leu Cys Gln Asn Gly Arg Cys Ser Asn Ile Val Pro Gly
                2760                2765                2770

TAC ATT TGC CTG TGC AAC CCT GGC TAC CAC TAT GAT GCC TCC AGC AGG      4608
Tyr Ile Cys Leu Cys Asn Pro Gly Tyr His Tyr Asp Ala Ser Ser Arg
            2775                2780                2785

AAG TGC CAG GAT CAC AAC GAA TGC CAG GAC TTG GCC TGT GAG AAC GGT      4656
Lys Cys Gln Asp His Asn Glu Cys Gln Asp Leu Ala Cys Glu Asn Gly
        2790                2795                2800
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GAG TGT GTG AAC CAA GAA GGC TCC TTC CAT TGC CTC TGC AAT CCC CCC     4704
Glu Cys Val Asn Gln Glu Gly Ser Phe His Cys Leu Cys Asn Pro Pro
    2805                2810                2815

CTC ACC CTA GAC CTC AGT GGG CAG CGC TGT GTG AAC ACG ACC AGC AGC     4752
Leu Thr Leu Asp Leu Ser Gly Gln Arg Cys Val Asn Thr Thr Ser Ser
2820                2825                2830                2835

ACG GAG GAC TTC CCT GAC CAT GAC ATC CAC ATG GAC ATC TGC TGG AAA     4800
Thr Glu Asp Phe Pro Asp His Asp Ile His Met Asp Ile Cys Trp Lys
                2840                2845                2850

AAA GTC ACC AAT GAT GTG TGC AGC CAG CCC TTG CGT GGG CAC CAT ACC     4848
Lys Val Thr Asn Asp Val Cys Ser Gln Pro Leu Arg Gly His His Thr
            2855                2860                2865

ACC TAT ACA GAA TGC TGC TGC CAA GAT GGG GAG GCC TGG AGC CAG CAA     4896
Thr Tyr Thr Glu Cys Cys Cys Gln Asp Gly Glu Ala Trp Ser Gln Gln
        2870                2875                2880

TGC GCT CTG TGC CCG CCC AGG AGC TCT GAG GTC TAC GCT CAG CTG TGC     4944
Cys Ala Leu Cys Pro Pro Arg Ser Ser Glu Val Tyr Ala Gln Leu Cys
    2885                2890                2895
```

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AAC GTG GCT CGG ATT GAG GCA GAG CGC GGA GCA GGG ATC CAC TTC CGG      4992
Asn Val Ala Arg Ile Glu Ala Glu Arg Gly Ala Gly Ile His Phe Arg
2900                5905                2910                2915

CCA GGC TAT GAG TAT GGC CCT GGC CTG GAC GAT CTG CCT GAA AAC CTC      5040
Pro Gly Tyr Glu Tyr Gly Pro Gly Leu Asp Asp Leu Pro Glu Asn Leu
                2920                2925                2930

TAC GGC CCA GAT GGG GCT CCC TTC TAT AAC TAC CTA GGC CCC GAG GAC      5088
Tyr Gly Pro Asp Gly Ala Pro Phe Tyr Asn Tyr Leu Gly Pro Glu Asp
            2935                2940                2945

ACT GCC CCT GAG CCT CCC TTC TCC AAC CCA GCC AGC CAG CCG GGA GAC      5136
Thr Ala Pro Glu Pro Pro Phe Ser Asn Pro Ala Ser Gln Pro Gly Asp
        2950                2955                2960

AAC ACA CCT GTC CTT GAG CCT CCT CTG CAG CCC TCT GAA CTT CAG CCT      5184
Asn Thr Pro Val Leu Glu Pro Pro Leu Gln Pro Ser Glu Leu Gln Pro
    2965                2970                2975

CAC TAT CTA GCC AGC CAC TCA GAA CCC CCT GCC TCC TTC GAA GGC CTT      5232
His Tyr Leu Ala Ser His Ser Glu Pro Pro Ala Ser Phe Glu Gly Leu
2980                2985                2990                2995
```

```
CAG GCT GAG GAA TGT GGC ATC CTG AAT GGC TGT GAG AAT GGC CGC TGC      5280
Gln Ala Glu Glu Cys Gly Ile Leu Asn Gly Cys Glu Asn Gly Arg Cys
                3000                3005                3010

GTG CGT GTG CGG GAG GGC TAC ACT TGC GAC TGC TTT GAG GGC TTC CAG      5328
Val Arg Val Arg Glu Gly Tyr Thr Cys Asp Cys Phe Glu Gly Phe Gln
            3015                3020                3025

CTG GAT GCG CCC ACA TTG GCC TGT GTG GAT GTG AAC GAG TGT GAA GAC      5376
Leu Asp Ala Pro Thr Leu Ala Cys Val Asp Val Asn Glu Cys Glu Asp
        3030                3035                3040

TTG AAC GGG CCT GCA CGA CTC TGT GCA CAC GGT CAC TGT GAG AAC ACA      5424
Leu Asn Gly Pro Ala Arg Leu Cys Ala His Gly His Cys Glu Asn Thr
    3045                3050                3055

GAG GGT TCC TAT CGC TGC CAC TGT TCG CCA GGT TAC GTG GCA GAG CCA      5472
Glu Gly Ser Tyr Arg Cys His Cys Ser Pro Gly Tyr Val Ala Glu Pro
3060                3065                3070                3075

GGC CCC CCA CAC TGT GCG GCC AAG GAG TAG                              5502
Gly Pro Pro His Cys Ala Ala Lys Glu  *
                3080                3085
```

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1834 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

```
Met Glu Ser Thr Ser Pro Arg Gly Leu Arg Cys Pro Gln Leu Cys Ser
 1               5                  10                  15

His Ser Gly Ala Met Arg Ala Pro Thr Thr Ala Arg Cys Ser Gly Cys
            20                  25                  30

Ile Gln Arg Val Arg Trp Arg Gly Phe Leu Pro Leu Val Leu Ala Val
        35                  40                  45

Leu Met Gly Thr Ser His Ala Gln Arg Asp Ser Ile Gly Arg Tyr Glu
    50                  55                  60
```

```
Pro Ala Ser Arg Asp Ala Asn Arg Leu Trp His Pro Val Gly Ser His
 65                  70                  75                  80

Pro Ala Ala Ala Ala Ala Lys Val Tyr Ser Leu Phe Arg Glu Pro Asp
                 85                  90                  95

Ala Pro Val Pro Gly Leu Ser Pro Ser Glu Trp Asn Gln Pro Ala Gln
            100                 105                 110

Gly Asn Pro Gly Trp Leu Ala Glu Ala Glu Ala Arg Arg Pro Pro Arg
        115                 120                 125

Thr Gln Gln Leu Arg Arg Val Gln Pro Pro Val Gln Thr Arg Arg Ser
    130                 135                 140

His Pro Arg Gly Gln Gln Gln Ile Ala Ala Arg Ala Ala Pro Ser Val
145                 150                 155                 160

Ala Arg Leu Glu Thr Pro Gln Arg Pro Ala Ala Ala Arg Arg Gly Arg
                165                 170                 175

Leu Thr Gly Arg Asn Val Cys Gly Gly Gln Cys Cys Pro Gly Trp Thr
            180                 185                 190
```

```
Thr Ser Asn Ser Thr Asn His Cys Ile Lys Pro Val Cys Gln Pro Pro
        195                 200                 205

Cys Gln Asn Arg Gly Ser Cys Ser Arg Pro Gln Val Cys Ile Cys Arg
    210                 215                 220

Ser Gly Phe Arg Gly Ala Arg Cys Glu Glu Val Ile Pro Glu Glu Glu
225                 230                 235                 240

Phe Asp Pro Gln Asn Ala Arg Pro Val Pro Arg Arg Ser Val Glu Arg
                245                 250                 255

Ala Pro Gly Pro His Arg Ser Ser Glu Ala Arg Gly Ser Leu Val Thr
            260                 265                 270

Arg Ile Gln Pro Leu Val Pro Pro Pro Ser Pro Pro Pro Ser Arg Arg
        275                 280                 285

Leu Ser Gln Pro Trp Pro Leu Gln Gln His Ser Gly Pro Ser Arg Thr
    290                 295                 300

Val Arg Arg Tyr Pro Ala Thr Gly Ala Asn Gly Gln Leu Met Ser Asn
305                 310                 315                 320
```

```
Ala Leu Pro Ser Gly Leu Glu Leu Arg Asp Ser Ser Pro Gln Ala Ala
                325                 330                 335

His Val Asn His Leu Ser Pro Pro Trp Gly Leu Asn Leu Thr Glu Lys
            340                 345                 350

Ile Lys Lys Ile Lys Val Val Phe Thr Pro Thr Ile Cys Lys Gln Thr
        355                 360                 365

Cys Ala Arg Gly Arg Cys Ala Asn Ser Cys Glu Lys Gly Asp Thr Thr
    370                 375                 380

Thr Leu Tyr Ser Gln Gly Gly His Gly His Asp Pro Lys Ser Gly Phe
385                 390                 395                     400

Arg Ile Tyr Phe Cys Gln Ile Pro Cys Leu Asn Gly Gly Arg Cys Ile
                405                 410                 415

Gly Arg Asp Glu Cys Trp Cys Pro Ala Asn Ser Thr Gly Lys Phe Cys
            420                 425                 430

His Leu Pro Val Pro Gln Pro Asp Arg Glu Pro Ala Gly Arg Gly Ser
        435                 440                 445
```

```
Arg His Arg Thr Leu Leu Glu Gly Pro Leu Lys Gln Ser Thr Phe Thr
    450                 455                 460

Leu Pro Leu Ser Asn Gln Leu Ala Ser Val Asn Pro Ser Leu Val Lys
465                 470                 475                 480

Val Gln Ile His His Pro Pro Glu Ala Ser Val Gln Ile His Gln Val
                485                 490                 495

Ala Arg Val Arg Gly Glu Leu Asp Pro Val Leu Glu Asp Asn Ser Val
            500                 505                 510

Glu Thr Arg Ala Ser His Arg Pro His Gly Asn Leu Gly His Ser Pro
        515                 520                 525

Trp Ala Ser Asn Ser Ile Pro Ala Arg Ala Gly Glu Ala Pro Arg Pro
    530                 535                 540

Pro Pro Val Leu Ser Arg His Tyr Gly Leu Leu Gly Gln Cys Tyr Leu
545                 550                 555                 560

Ser Thr Val Asn Gly Gln Cys Ala Asn Pro Leu Gly Ser Leu Thr Ser
                565                 570                 575
```

```
Gln Glu Asp Cys Cys Gly Ser Val Gly Thr Phe Trp Gly Val Thr Ser
            580                 585                 590

Cys Ala Pro Cys Pro Pro Arg Gln Glu Gly Pro Ala Phe Pro Val Ile
        595                 600                 605

Glu Asn Gly Gln Leu Glu Cys Pro Gln Gly Tyr Lys Arg Leu Asn Leu
    610                 615                 620

Ser His Cys Gln Asp Ile Asn Glu Cys Leu Thr Leu Gly Leu Cys Lys
625                 630                 635                 640

Asp Ser Glu Cys Val Asn Thr Arg Gly Ser Tyr Leu Cys Thr Cys Arg
                645                 650                 655

Pro Gly Leu Met Leu Asp Pro Ser Arg Ser Arg Cys Val Ser Asp Lys
            660                 665                 670

Ala Val Ser Met Gln Gln Gly Leu Cys Tyr Arg Ser Leu Gly Ser Gly
        675                 680                 685

Thr Cys Thr Leu Pro Leu Val His Arg Ile Thr Lys Gln Ile Cys Cys
    690                 695                 700
```

```
Cys Ser Arg Val Gly Lys Ala Trp Gly Ser Thr Cys Glu Gln Cys Pro
705                 710                 715                 720

Leu Pro Gly Thr Glu Ala Phe Arg Glu Ile Cys Pro Ala Gly His Gly
                725                 730                 735

Tyr Thr Tyr Ser Ser Ser Asp Ile Arg Leu Ser Met Arg Lys Ala Glu
            740                 745                 750

Glu Glu Glu Leu Ala Ser Pro Leu Arg Glu Gln Thr Glu Gln Ser Thr
        755                 760                 765

Ala Pro Pro Pro Gly Gln Ala Glu Arg Gln Pro Leu Arg Ala Ala Thr
    770                 775                 780

Ala Thr Trp Ile Glu Ala Glu Thr Leu Pro Asp Lys Gly Asp Ser Arg
785                 790                 795                 800

Ala Val Gln Ile Thr Thr Ser Ala Pro His Leu Pro Ala Arg Val Pro
                805                 810                 815

Gly Asp Ala Thr Gly Arg Pro Ala Pro Ser Leu Pro Gly Gln Gly Ile
            820                 825                 830
```

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Pro Glu Ser Pro Ala Glu Glu Gln Val Ile Pro Ser Ser Asp Val Leu
        835                 840                 845

Val Thr His Ser Pro Pro Asp Phe Asp Pro Cys Phe Ala Gly Ala Ser
    850                 855                 860

Asn Ile Cys Gly Pro Gly Thr Cys Val Ser Leu Pro Asn Gly Tyr Arg
865                 870                 875                 880

Cys Val Cys Ser Pro Gly Tyr Gln Leu His Pro Ser Gln Asp Tyr Cys
                885                 890                 895

Thr Asp Asp Asn Glu Cys Met Arg Asn Pro Cys Glu Gly Arg Gly Arg
            900                 905                 910

Cys Val Asn Ser Val Gly Ser Tyr Ser Cys Leu Cys Tyr Pro Gly Tyr
        915                 920                 925

Thr Leu Val Thr Leu Gly Asp Thr Gln Glu Cys Gln Asp Ile Asp Glu
    930                 935                 940

Cys Glu Gln Pro Gly Val Cys Ser Gly Gly Arg Cys Ser Asn Thr Glu
945                 950                 955                 960
```

```
Gly Ser Tyr His Cys Glu Cys Asp Arg Gly Tyr Ile Met Val Arg Lys
                965                 970                 975

Gly His Cys Gln Asp Ile Asn Glu Cys Arg His Pro Gly Thr Cys Pro
            980                 985                 990

Asp Gly Arg Cys Val Asn Ser Pro Gly Ser Tyr Thr Cys Leu Ala Cys
        995                 1000                1005

Glu Glu Gly Tyr Val Gly Gln Ser Gly Ser Cys Val Asp Val Asn Glu
    1010                1015                1020

Cys Leu Thr Pro Gly Ile Cys Thr His Gly Arg Cys Ile Asn Met Glu
1025                1030                1035                1040

Gly Ser Phe Arg Cys Ser Cys Glu Pro Gly Tyr Glu Val Thr Pro Asp
                1045                1050                1055

Lys Lys Gly Cys Arg Asp Val Asp Glu Cys Ala Ser Arg Ala Ser Cys
            1060                1065                1070

Pro Thr Gly Leu Cys Leu Asn Thr Glu Gly Ser Phe Thr Cys Ser Ala
        1075                1080                1085
```

```
Cys Gln Ser Gly Tyr Trp Val Asn Glu Asp Gly Thr Ala Cys Glu Asp
    1090                1095                1100

Leu Asp Glu Cys Ala Phe Pro Gly Val Cys Pro Thr Gly Val Cys Thr
1105                1110                1115                1120

Asn Thr Val Gly Ser Phe Ser Cys Lys Asp Cys Asp Gln Gly Tyr Arg
                1125                1130                1135

Pro Asn Pro Leu Gly Asn Arg Cys Glu Asp Val Asp Glu Cys Glu Gly
            1140                1145                1150

Pro Gln Ser Ser Cys Arg Gly Gly Glu Cys Lys Asn Thr Glu Gly Ser
        1155                1160                1165

Tyr Gln Cys Leu Cys His Gln Gly Phe Gln Leu Val Asn Gly Thr Met
    1170                1175                1180

Cys Glu Asp Val Asn Glu Cys Val Gly Glu Glu His Cys Ala Pro His
1185                1190                1195                1200

Gly Glu Cys Leu Asn Ser Leu Gly Ser Phe Phe Cys Leu Cys Ala Pro
                1205                1210                1215
```

```
Gly Phe Ala Ser Ala Glu Gly Gly Thr Arg Cys Gln Asp Val Asp Glu
            1220                1225                1230

Cys Ala Ala Thr Asp Pro Cys Pro Gly Gly His Cys Val Asn Thr Glu
        1235                1240                1245

Gly Ser Phe Ser Cys Leu Cys Glu Thr Ala Ser Phe Gln Pro Ser Pro
    1250                1255                1260

Asp Ser Gly Glu Cys Leu Asp Ile Asp Glu Cys Glu Asp Arg Glu Asp
1265                1270                1275                1280

Pro Val Cys Gly Ala Trp Arg Cys Glu Asn Ser Pro Gly Ser Tyr Arg
                1285                1290                1295

Cys Ile Leu Asp Cys Gln Pro Gly Phe Tyr Val Ala Pro Asn Gly Asp
            1300                1305                1310

Cys Ile Asp Ile Asp Glu Cys Ala Asn Asp Thr Val Cys Gly Asn His
        1315                1320                1325

Gly Phe Cys Asp Asn Thr Asp Gly Ser Phe Arg Cys Leu Cys Asp Gln
    1330                1335                1340
```

```
Gly Phe Glu Thr Ser Pro Ser Gly Trp Glu Cys Val Asp Val Asn Glu
1345                1350                1355                1360

Cys Glu Leu Met Met Ala Val Cys Gly Asp Ala Leu Cys Glu Asn Val
                1365                1370                1375

Glu Gly Ser Phe Leu Cys Leu Cys Ala Ser Asp Leu Glu Glu Tyr Asp
            1380                1385                1390

Ala Glu Glu Gly His Cys Arg Pro Arg Val Ala Gly Ala Gln Arg Ile
        1395                1400                1405

Pro Glu Val Arg Thr Glu Asp Gln Ala Pro Ser Leu Ile Arg Met Glu
    1410                1415                1420

Cys Tyr Ser Glu His Asn Gly Gly Pro Pro Cys Ser Gln Ile Leu Gly
1425                1430                1435                1440

Gln Asn Ser Thr Gln Ala Glu Cys Cys Cys Thr Gln Gly Ala Arg Trp
                1445                1450                1455

Gly Lys Ala Cys Ala Pro Cys Pro Ser Glu Asp Ser Val Glu Phe Ser
            1460                1465                1470
```

```
Gln Leu Cys Pro Ser Gly Gln Gly Tyr Ile Pro Val Glu Gly Ala Trp
        1475                1480                1485

Thr Phe Gly Gln Thr Met Tyr Thr Asp Ala Asp Glu Cys Val Leu Phe
    1490                1495                1500

Gly Pro Ala Leu Cys Gln Asn Gly Arg Cys Ser Asn Ile Val Pro Gly
1505                1510                1515                1520

Tyr Ile Cys Leu Cys Asn Pro Gly Tyr His Tyr Asp Ala Ser Ser Arg
                1525                1530                1535

Lys Cys Gln Asp His Asn Glu Cys Gln Asp Leu Ala Cys Glu Asn Gly
            1540                1545                1550

Glu Cys Val Asn Gln Glu Gly Ser Phe His Cys Leu Cys Asn Pro Pro
        1555                1560                1565

Leu Thr Leu Asp Leu Ser Gly Gln Arg Cys Val Asn Thr Thr Ser Ser
    1570                1575                1580

Thr Glu Asp Phe Pro Asp His Asp Ile His Met Asp Ile Cys Trp Lys
1585                1590                1595                1600
```

```
Lys Val Thr Asn Asp Val Cys Ser Gln Pro Leu Arg Gly His His Thr
                1605                1610                1615

Thr Tyr Thr Glu Cys Cys Cys Gln Asp Gly Glu Ala Trp Ser Gln Gln
            1620                1625                1630

Cys Ala Leu Cys Pro Pro Arg Ser Ser Glu Val Tyr Ala Gln Leu Cys
        1635                1640                1645

Asn Val Ala Arg Ile Glu Ala Glu Arg Gly Ala Gly Ile His Phe Arg
    1650                1655                1660

Pro Gly Tyr Glu Tyr Gly Pro Gly Leu Asp Asp Leu Pro Glu Asn Leu
1665                1670                1675                1680

Tyr Gly Pro Asp Gly Ala Pro Phe Tyr Asn Tyr Leu Gly Pro Glu Asp
                1685                1690                1695

Thr Ala Pro Glu Pro Pro Phe Ser Asn Pro Ala Ser Gln Pro Gly Asp
            1700                1705                1710

Asn Thr Pro Val Leu Glu Pro Pro Leu Gln Pro Ser Glu Leu Gln Pro
        1715                1720                1725
```

```
His Tyr Leu Ala Ser His Ser Glu Pro Pro Ala Ser Phe Glu Gly Leu
    1730                1735                1740

Gln Ala Glu Glu Cys Gly Ile Leu Asn Gly Cys Glu Asn Gly Arg Cys
1745                1750                1755                1760

Val Arg Val Arg Glu Gly Tyr Thr Cys Asp Cys Phe Glu Gly Phe Gln
                1765                1770                1775

Leu Asp Ala Pro Thr Leu Ala Cys Val Asp Val Asn Glu Cys Glu Asp
            1780                1785                1790

Leu Asn Gly Pro Ala Arg Leu Cys Ala His Gly His Cys Glu Asn Thr
        1795                1800                1805

Glu Gly Ser Tyr Arg Cys His Cys Ser Pro Gly Tyr Val Ala Glu Pro
    1810                1815                1820

Gly Pro Pro His Cys Ala Ala Lys Glu  *
1825                1830
```

CLAIMS

1. A method for transferring a nucleic acid segment into bone progenitor cells, comprising contacting bone progenitor cells with a composition comprising an isolated nucleic acid segment so as to transfer said nucleic acid segment into said cells.

2. The method of claim 1, wherein said cells are located within a bone progenitor tissue site of an animal and said tissue site is contacted with said composition so as to promote nucleic acid transfer into bone progenitor cells *in situ*.

3. The method of claim 2, wherein the contacting process comprises bringing said isolated nucleic acid segment into contact with a bone-compatible matrix to form a matrix-nucleic acid segment composition and bringing said matrix-nucleic acid segment composition into contact with said tissue site.

4. The use of a composition comprising an isolated nucleic acid segment and a bone-compatible matrix in the preparation of a formulation or medicament for transferring a nucleic acid segment into bone progenitor cells.

5. A use according to claim 4, wherein said formulation or medicament is intended for use in transferring a nucleic acid segment into bone progenitor cells within a bone progenitor tissue site of an animal.

6. A use according to claim 5, wherein said formulation or medicament is prepared by bringing an isolated nucleic acid segment into contact with a bone-compatible matrix to form a matrix-nucleic acid segment formulation or medicament intended for use in transferring a nucleic acid segment into bone progenitor cells within a bone progenitor tissue site of an animal.

7. A use according to claim 6, wherein said formulation or medicament is prepared by bringing an isolated nucleic acid segment into contact with a bone-compatible matrix and a pluronic agent to form a syringeable matrix-nucleic acid segment formulation or medicament.

8. A use according to claim 6, wherein said formulation or medicament further comprises a detectable agent for use in an imaging modality.

9. A use according to claim 8, wherein said formulation or medicament further comprises a radiographic agent.

10. A use according to claim 8, wherein said formulation or medicament further comprises a paramagnetic ion.

11. A use according to claim 8, wherein said formulation or medicament further comprises a radioactive ion.

12. A use according to claim 4, wherein said nucleic acid segment is a DNA molecule.

13. A use according to claim 4, wherein said nucleic acid segment is an RNA molecule.

14. A use according to claim 4, wherein said nucleic acid segment is an antisense nucleic acid molecule.

15. A use according to claim 4, wherein said nucleic acid segment is a linear nucleic acid molecule, a plasmid, a recombinant insert within the genome of a recombinant virus, or a nucleic acid segment associated with a liposome.

16. A use according to claim 15, wherein said nucleic acid segment is a nucleic acid segment associated with a liposome.

17. A use according to claim 4, wherein said nucleic acid segment encodes a polypeptide or protein that stimulates bone progenitor cells when expressed in said cells.

18. A use according to claim 6, wherein said bone-compatible matrix is a collagenous, metal, hydroxylapatite, bioglass, aluminate, bioceramic, acrylic ester polymer or lactic acid polymer matrix.

19. A use according to claim 18, wherein said bone-compatible matrix is a titanium matrix.

20. A use according to claim 19, wherein said bone-compatible matrix is a titanium matrix coated with hydroxylapatite.

21. A use according to claim 18, wherein said bone-compatible matrix is a collagen preparation.

22. A use according to claim 21, wherein said bone-compatible matrix is a type II collagen preparation.

23. A use according to claim 22, wherein said bone-compatible matrix is a type II collagen preparation obtained from hyaline cartilage.

24. A use according to claim 22, wherein said bone-compatible matrix is a recombinant type II collagen preparation.

25. A use according to claim 22, wherein said bone-compatible matrix is a mineralized type II collagen preparation.

26. A method of stimulating bone progenitor cells, comprising contacting bone progenitor cells with a composition comprising an isolated osteotropic gene so as to promote expression of said gene in said cells.

27. The method of claim 26, wherein said cells are located within a bone progenitor tissue site of an animal

and said tissue site is contacted with said composition so as to promote bone tissue growth.

28. The method of claim 27, wherein the contacting process comprises bringing said osteotropic gene into contact with a bone-compatible matrix to form a matrix-gene composition and bringing said matrix-gene composition into contact with said tissue site.

29. The use of a composition comprising an isolated osteotropic gene in the preparation of a formulation or medicament for use in promoting expression of the gene in bone progenitor cells and for stimulating said bone progenitor cells.

30. A use according to claim 29, wherein said formulation or medicament is intended for use in promoting expression of the gene in bone progenitor cells within a bone progenitor tissue site of an animal and for stimulating said bone progenitor cells to promote bone tissue growth.

31. A use according to claim 30, wherein said formulation or medicament is prepared by bringing an osteotropic gene into contact with a bone-compatible matrix to form a matrix-gene formulation or medicament intended for use in promoting expression of the gene in bone progenitor cells within a bone progenitor tissue site of an animal and for stimulating said bone progenitor cells to promote bone tissue growth.

32. A use according to claim 31, wherein said formulation or medicament is prepared by bringing an osteotropic gene into contact with a bone-compatible matrix and a pluronic agent to form a syringeable matrix-gene formulation or medicament.

33. A use according to claim 31, wherein said formulation or medicament further comprises a detectable agent for use in an imaging modality.

34. A use according to claim 33, wherein said formulation or medicament further comprises a radiographic agent.

35. A use according to claim 34, wherein said formulation or medicament further comprises calcium phosphate.

36. A use according to claim 33, wherein said formulation or medicament further comprises a paramagnetic ion.

37. A use according to claim 36, wherein said formulation or medicament further comprises chromium (III), manganese (II), iron (III), iron (II), cobalt (II), nickel (II), copper (II), neodymium (III), samarium (III), ytterbium (III), gadolinium (III), vanadium (II), terbium (III), dysprosium (III), holmium (III) or erbium (III).

38. A use according to claim 33, wherein said formulation or medicament further comprises a radioactive ion.

39. A use according to claim 38, wherein said formulation or medicament further comprises iodine^{131}, iodine^{123}, technicium^{99m}, indium^{111}, rhenium^{188}, rhenium^{186}, gallium^{67}, copper^{67}, yttrium^{90}, iodine^{125} or astatine^{211}.

40. A use according to claim 29, wherein said osteotropic gene is in the form of plasmid DNA, a DNA insert within the genome of a recombinant adenovirus, a DNA insert within the genome of a recombinant adeno-associated virus (AAV), a DNA insert within the genome of a recombinant retrovirus, or a DNA segment associated with a liposome.

41. A use according to claim 40, wherein said osteotropic gene is in the form of an osteotropic gene associated with a liposome.

42. A use according to claim 29, wherein said osteotropic gene is a parathyroid hormone (PTH) gene, a bone morphogenetic protein (BMP) gene, a growth factor gene, a growth factor receptor gene, a cytokine gene or a chemotactic factor gene.

43. A use according to claim 42, wherein said osteotropic gene is a transforming growth factor (TGF) gene, a fibroblast growth factor (FGF) gene, a granulocyte/macrophage colony stimulating factor (GMCSF)

gene, an epidermal growth factor (EGF) gene, a platelet derived growth factor (PDGF) gene, an insulin-like growth factor (IGF) gene, or a leukemia inhibitory factor (LIF) gene.

44. A use according to claim 43, wherein said osteotropic gene is a TGF-α, TGF-β1 or TGF-β2 gene.

45. A use according to claim 42, wherein said osteotropic gene is a PTH gene.

46. A use according to claim 42, wherein said osteotropic gene is a BMP gene.

47. A use according to claim 46, wherein said osteotropic gene is a BMP-2 or BMP-4 gene.

48. A use according to claim 31, wherein said bone-compatible matrix is a collagenous, metal, hydroxylapatite, bioglass, aluminate, bioceramic, acrylic ester polymer or lactic acid polymer matrix.

49. A use according to claim 48, wherein said bone-compatible matrix is a titanium matrix.

50. A use according to claim 49, wherein said bone-compatible matrix is a titanium matrix coated with hydroxylapatite.

51. A use according to claim 48, wherein said bone-compatible matrix is a collagen preparation.

52. A use according to claim 51, wherein said bone-compatible matrix is a type II collagen preparation.

53. A use according to claim 52, wherein said bone-compatible matrix is a type II collagen preparation obtained from hyaline cartilage.

54. A use according to claim 52, wherein said bone-compatible matrix is a recombinant type II collagen preparation.

55. A use according to claim 52, wherein said bone-compatible matrix is a mineralized type II collagen preparation.

56. A use according to claim 31, wherein said matrix-gene composition is applied to a bone fracture site in said animal.

57. A use according to claim 31, wherein said matrix-gene composition is implanted within a bone cavity site in said animal.

58. A use according to claim 31, wherein said bone cavity site is the result of dental or periodontal surgery or the removal of an osteosarcoma.

59. A composition comprising an isolated nucleic acid segment in association with a bone-compatible matrix.

60. The composition of claim 59, wherein said nucleic acid segment is a DNA molecule.

61. The composition of claim 59, wherein said nucleic acid segment is an RNA molecule.

62. The composition of claim 59, wherein said nucleic acid segment is an antisense nucleic acid molecule.

63. The composition of claim 59, wherein said nucleic acid segment is a linear nucleic acid molecule, a plasmid, a recombinant insert within the genome of a recombinant virus, or a nucleic acid segment associated with a liposome.

64. The composition of claim 63, wherein said nucleic acid segment is associated with a liposome.

65. The composition of claim 59, wherein said nucleic acid segment encodes a polypeptide or protein that stimulates bone progenitor cells when expressed in said cells.

66. The composition of claim 59, wherein said bone-compatible matrix is a collagenous, titanium, hydroxylapatite, hydroxylapatite-coated titanium, bioglass, aluminate, bioceramic, acrylic ester polymer or

lactic acid polymer matrix.

67. The composition of claim 66, wherein said bone-compatible matrix is a collagen preparation.

68. The composition of claim 67, wherein said bone-compatible matrix is a type II collagen preparation.

69. The composition of claim 68, wherein said bone-compatible matrix is a type II collagen preparation obtained from hyaline cartilage.

70. The composition of claim 68, wherein said bone-compatible matrix is a recombinant type II collagen preparation.

71. The composition of claim 68, wherein said bone-compatible matrix is a mineralized type II collagen preparation.

72. The composition of claim 59, further defined as a syringeable composition.

73. The composition of claim 59, wherein said composition further comprises a detectable agent for use in an imaging modality.

74. The composition of claim 73, wherein said composition further comprises a radiographic agent.

75. The composition of claim 73, wherein said composition further comprises a paramagnetic ion.

76. The composition of claim 73, wherein said composition further comprises a radioactive ion.

77. A composition comprising an isolated osteotropic gene in association with a bone-compatible matrix, said composition being capable of stimulating bone growth when administered to a bone progenitor tissue site of an animal.

78. The composition of claim 77, wherein said osteotropic gene is in the form of plasmid DNA, a DNA insert within the genome of a recombinant adenovirus, a DNA insert within the genome of a recombinant adeno-associated virus (AAV), a DNA insert within the genome of a recombinant retrovirus, or a DNA segment associated with a liposome.

79. The composition of claim 78, wherein said osteotropic gene is in the form of an osteotropic gene associated with a liposome.

80. The composition of claim 77, wherein said osteotropic gene is a PTH, BMP, TGF-α, TGF-β1, TGF-β2, FGF, GMCSF, EGF, PDGF, IGF or a LIF gene.

81. The composition claim 80, wherein said osteotropic gene is a TGF-α, TGF-β1, TGF-β2, PTH, BMP-2 or BMP-4 gene.

82. The composition of claim 77, wherein said bone-compatible matrix is a collagenous, metal, hydroxyapatite, bioglass, aluminate, bioceramic, acrylic ester polymer or lactic acid polymer matrix.

83. The composition of claim 82, wherein said bone-compatible matrix is a titanium matrix.

84. The composition of claim 83, wherein said bone-compatible matrix is a titanium matrix coated with hydroxylapatite.

85. The composition of claim 82, wherein said bone-compatible matrix is a collagen preparation.

86. The composition of claim 85, wherein said bone-compatible matrix is a type II collagen preparation.

87. The composition of claim 86, wherein said bone-compatible matrix is a type II collagen preparation obtained from hyaline cartilage.

88. The composition of claim 86, wherein said bone-compatible matrix is a recombinant type II collagen preparation.

89. The composition of claim 86, wherein said bone-compatible matrix is a mineralized type II collagen preparation.

90. The composition of claim 77, further defined as comprising an isolated osteotropic gene in association with a bone-compatible matrix and a pluronic agent, the composition forming a syringeable composition.

91. The composition of claim 77, wherein said composition further comprises a detectable agent for use in an imaging modality.

92. The composition of claim 91, wherein said composition further comprises a radiographic agent.

93. The composition of claim 92, wherein said composition further comprises calcium phosphate.

94. The composition of claim 91, wherein said composition further comprises a paramagnetic ion.

95. The composition of claim 94, wherein said composition further comprises chromium (III), manganese (II), iron (III), iron (II), cobalt (II), nickel (II), copper (II), neodymium (III), samarium (III), ytterbium (III), gadolinium (III), vanadium (II), terbium (III), dysprosium (III), holmium (III) or erbium (III).

96. The composition of claim 91, wherein said composition further comprises a radioactive ion.

97. The composition of claim 96, wherein said composition further comprises iodine^{131}, iodine^{123}, technicium^{99m}, indium^{111}, rhenium^{188}, rhenium^{186}, gallium^{67}, copper^{67}, yttrium^{90}, iodine^{125} or astatine^{211}.

98. A kit comprising, in suitable container means, a pharmaceutically acceptable bone-compatible matrix and a pharmaceutically acceptable osteotropic gene preparation.

99. The kit of claim 98, wherein said bone-compatible matrix is a collagenous, titanium, hydroxylapatite, hydroxylapatite-coated titanium, bioglass, aluminate, bioceramic, acrylic ester polymer or lactic acid polymer matrix.

100. The kit of claim 99, wherein said bone-compatible matrix is a titanium matrix.

101. The kit of claim 99, wherein said bone-compatible matrix is a hydroxylapatite-coated titanium matrix.

102. The kit of claim 99, wherein said bone-compatible matrix is a collagenous matrix.

103. The kit of claim 102, wherein said bone-compatible matrix is a type II collagen matrix.

104. The kit of claim 103, wherein said bone-compatible matrix is a type II collagen matrix obtained from hyaline cartilage.

105. The kit of claim 103, wherein said bone-compatible matrix is a recombinant type II collagen matrix.

106. The kit of claim 103, wherein said bone-compatible matrix is a mineralized type II collagen matrix.

107. The kit of claim 98, wherein said osteotropic gene preparation comprises a linear osteotropic gene, a plasmid including an osteotropic gene, a recombinant virus having a genome that includes an osteotropic gene or an osteotropic gene associated with a liposome.

108. The kit of claim 98, wherein said osteotropic gene preparation comprises a lyophilized gene preparation.

109. The kit of claim 98, wherein said osteotropic gene preparation comprises a PTH, TGF, BMP, FGF, GMCSF, EGF, PDGF, IGF or a LIF gene.

110. The kit of claim 109, wherein said osteotropic gene preparation comprises a PTH, TGF-ß1, TGF-ß2, TGF-ß3, BMP-2 or a BMP-4 gene.

111. The kit of claim 98, further comprising a pluronic agent.

112. The kit of claim 98, further comprising a detectable agent for use in an imaging modality.

113. The kit of claim 112, wherein said composition further comprises a radiographic agent.

114. The kit of claim 113, wherein said composition further comprises calcium phosphate.

115. The kit of claim 112, wherein said composition further comprises a paramagnetic ion.

116. The kit of claim 115, wherein said composition further comprises chromium (III), manganese (II), iron (III), iron (II), cobalt (II), nickel (II), copper (II), neodymium (III), samarium (III), ytterbium (III), gadolinium (III), vanadium (II), terbium (III), dysprosium (III), holmium (III) or erbium (III).

117. The kit of claim 112, wherein said composition further comprises a radioactive ion.

118. The kit of claim 117, wherein said composition further comprises $iodine^{131}$, $iodine^{123}$, $technicium^{99m}$, $indium^{111}$, $rhenium^{188}$, $rhenium^{186}$, $gallium^{67}$, $copper^{67}$, $yttrium^{90}$, $iodine^{125}$ or $astatine^{211}$.

119. The kit of claim 98, wherein said bone-compatible

matrix and said osteotropic gene preparation are present within a single container means.

120. The kit of claim 119, wherein said container means is a syringe or pipette.

121. The kit of claim 98, wherein said bone-compatible matrix and said osteotropic gene preparation are present within distinct container means.

122. The kit of claim 98, further comprising a third container means comprising a pharmaceutically acceptable diluent.

123. The kit of claim 98, further comprising a syringe, pipette or forceps.

124. An osteotropic device, comprising an isolated osteotropic gene capable of expression in bone progenitor cells, the gene associated with an amount of a bone-compatible matrix effective to absorb said gene, wherein said device is capable of stimulating bone formation when implanted within a bone progenitor tissue site of an animal.

125. The device of claim 124, wherein said device is a titanium or a hydroxylapatite-coated titanium device.

126. The device of claim 124, wherein said device is shaped to join a bone fracture site in said animal.

127. The device of claim 124, wherein said device is shaped to fill a bone cavity site in said animal.

128. The device of claim 124, wherein said device is an artificial joint.

129. A DNA segment comprising an isolated gene that encodes a protein or peptide that includes an amino acid sequence essentially as set forth by a contiguous sequence from SEQ ID NO:3.

130. The DNA segment of claim 129, comprising an isolated gene that includes a nucleic acid sequence essentially as set forth by a contiguous sequence from SEQ ID NO:2.

131. A composition comprising a purified protein that has an amino acid sequence essentially as set forth by the amino acid sequence of SEQ ID NO:3.

132. A method for stimulating a bone progenitor cell, comprising contacting a bone progenitor cell with a composition comprising a biologically effective amount of type II collagen.

133. The use of a composition comprising a biologically effective amount of type II collagen in the preparation of a formulation or medicament for stimulating a bone progenitor cell.

134. A use according to claim 133, wherein said composition comprises type II collagen purified from hyaline cartilage.

135. A use according to claim 133, wherein said composition comprises recombinant type II collagen.

136. A use according to claim 133, wherein said composition comprises type II collagen further supplemented with minerals.

137. A use according to claim 136, wherein said composition comprises type II collagen further supplemented with calcium.

138. A use according to claim 133, wherein said composition comprises between about 1 mg and about 500 mg of type II collagen.

139. A use according to claim 138, wherein said composition comprises between about 1 mg and about 100 mg of type II collagen.

140. A use according to claim 139, wherein said composition comprises about 10 mg of type II collagen.

141. A use according to claim 133, wherein said composition comprises type II collagen in combination with a nucleic acid segment that encodes a polypeptide or

protein that stimulates bone progenitor cells when expressed in said cells.

142. A use according to claim 141, wherein said nucleic acid segment comprises an isolated PTH gene, BMP gene, growth factor gene, growth factor receptor gene, cytokine gene or chemotactic factor gene.

143. A use according to claim 142, wherein said nucleic acid segment comprises an isolated BMP gene.

144. A use according to claim 143, wherein said nucleic acid segment comprises an isolated BMP-2 or BMP-4 gene.

145. A use according to claim 141, wherein said composition further comprises a detectable agent for use in an imaging modality.

146. A use according to claim 133, wherein said formulation or medicament is intended for use in stimulating a bone progenitor cell located within a bone progenitor tissue site of an animal and for promoting bone tissue growth.

147. A use according to claim 146, wherein said formulation or medicament is intended for use in implantation within a bone cavity site in an animal and for promoting bone tissue growth in said bone cavity site.

148. A use according to claim 146, wherein said formulation or medicament is intended for use in implantation within a bone fracture site in an animal and for promoting bone tissue growth in said bone fracture site.

149. A method for promoting bone growth, comprising contacting a bone progenitor tissue site of an animal with a composition comprising type II collagen in an amount effective to activate bone progenitor cells of said tissue site.

150. The use of a composition comprising a biologically effective amount of type II collagen in the preparation of a formulation or medicament for promoting bone growth in a bone progenitor tissue site of an animal.

151. A use according to claim 150, wherein said composition comprises recombinant type II collagen.

152. A use according to claim 150, wherein said composition comprises type II collagen further supplemented with minerals.

153. A use according to claim 150, wherein said composition comprises type II collagen and an osteotropic gene in a combined amount effective to activate bone progenitor cells of said tissue site.

154. A use according to claim 153, wherein said composition comprises type II collagen in combination with a PTH, TGF-ß or BMP gene.

155. A use according to claim 153, wherein said composition further comprises a detectable agent for use in an imaging modality.

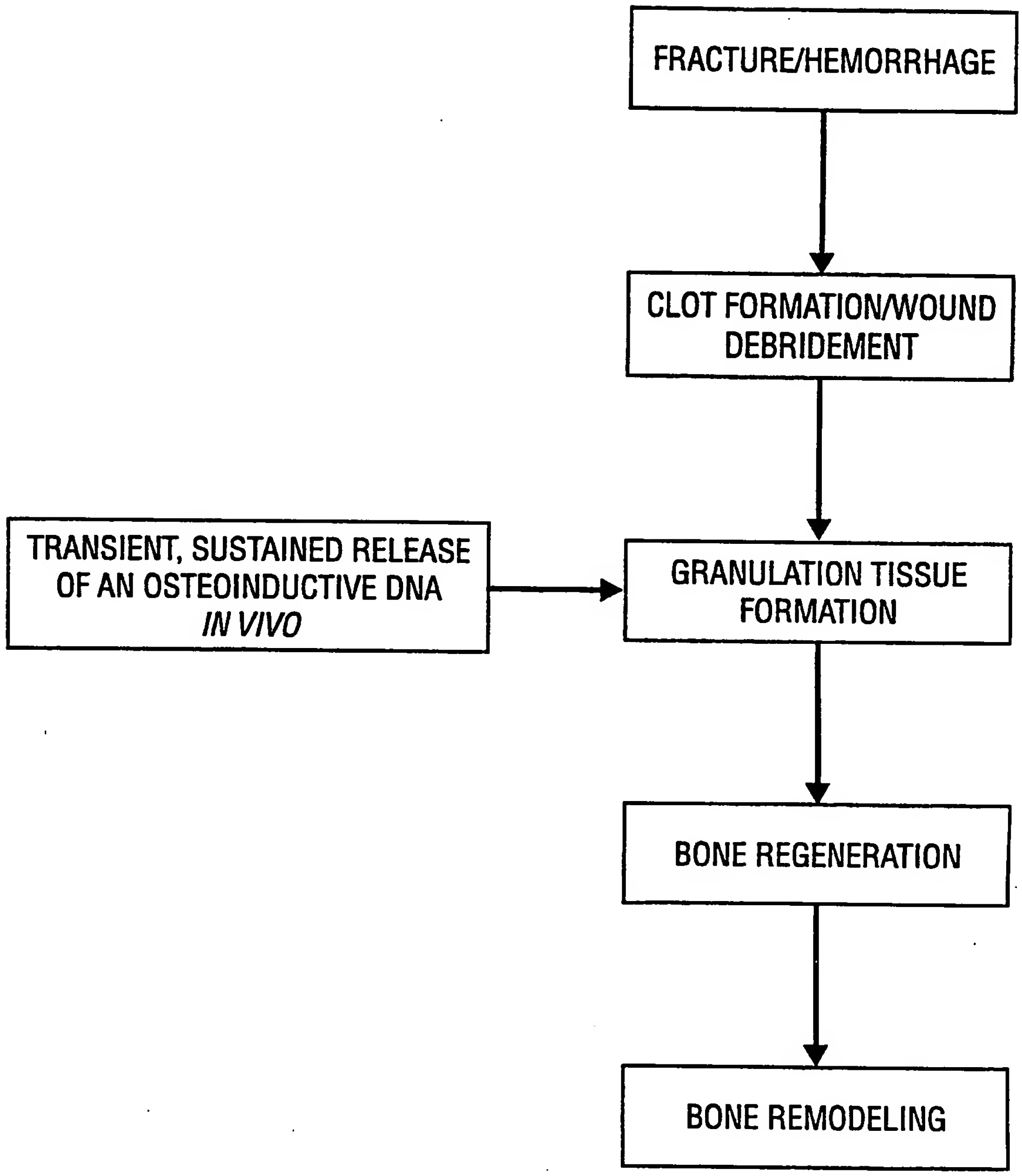

FRACTURE/HEMORRHAGE
CLOT FORMATION/WOUND DEBRIDEMENT
TRANSIENT, SUSTAINED RELEASE OF AN OSTEOINDUCTIVE DNA *IN VIVO*
GRANULATION TISSUE FORMATION
BONE REGENERATION
BONE REMODELING

FIG. 1

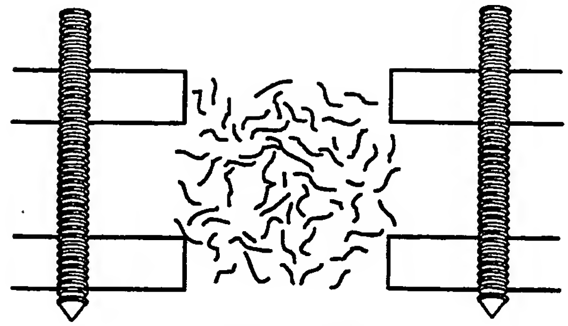


FIG. 2A

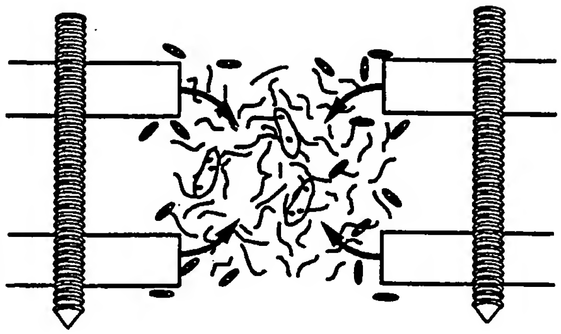


FIG. 2B

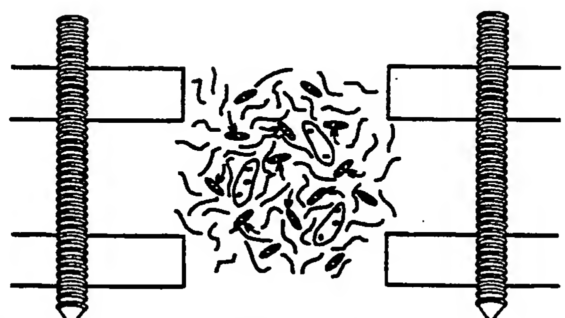


FIG. 2C

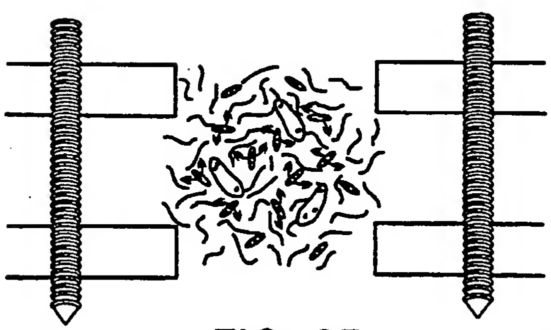


FIG. 2D

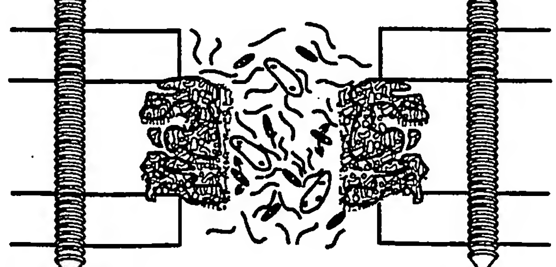


FIG. 2E

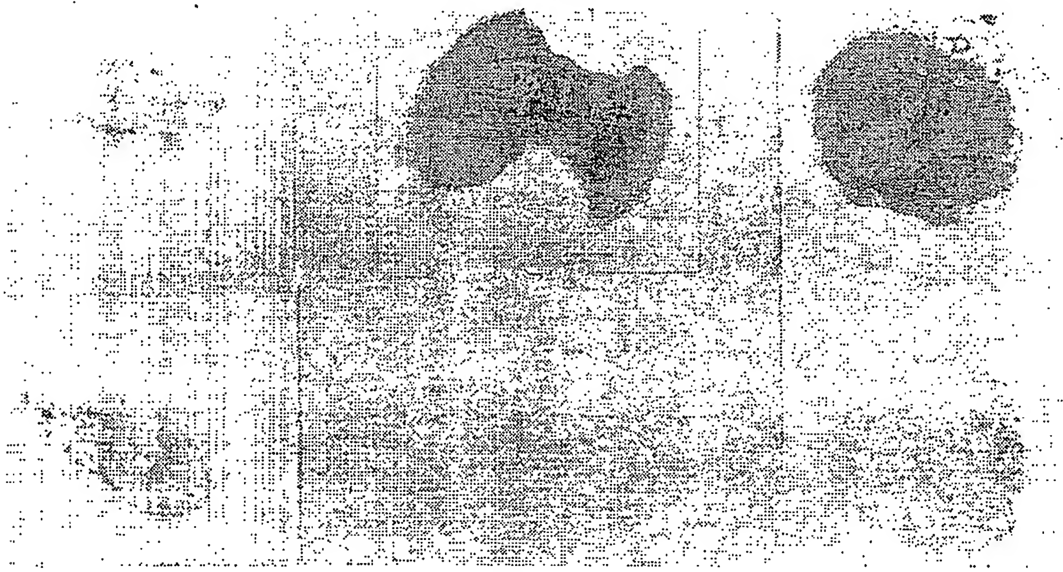


FIG. 3A FIG. 3B FIG. 3C

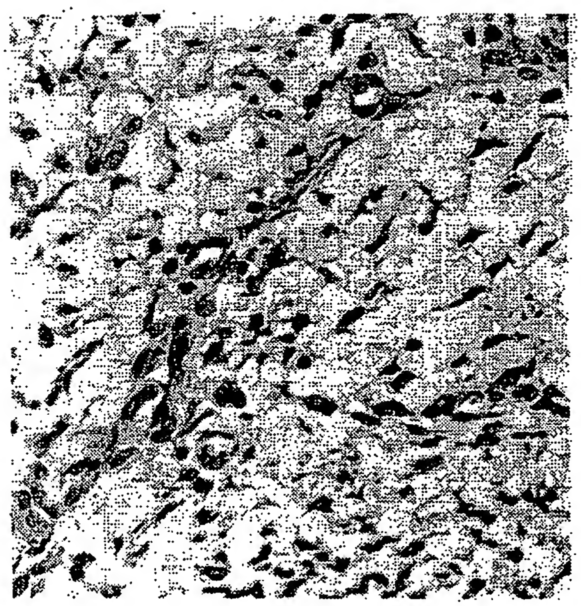


FIG.3D

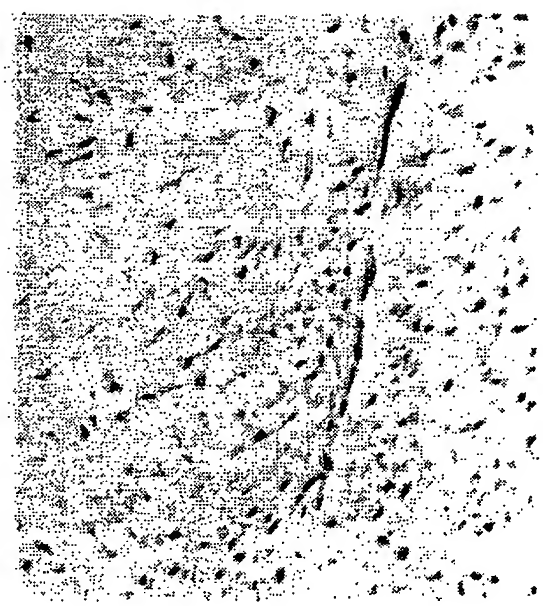


FIG. 3E

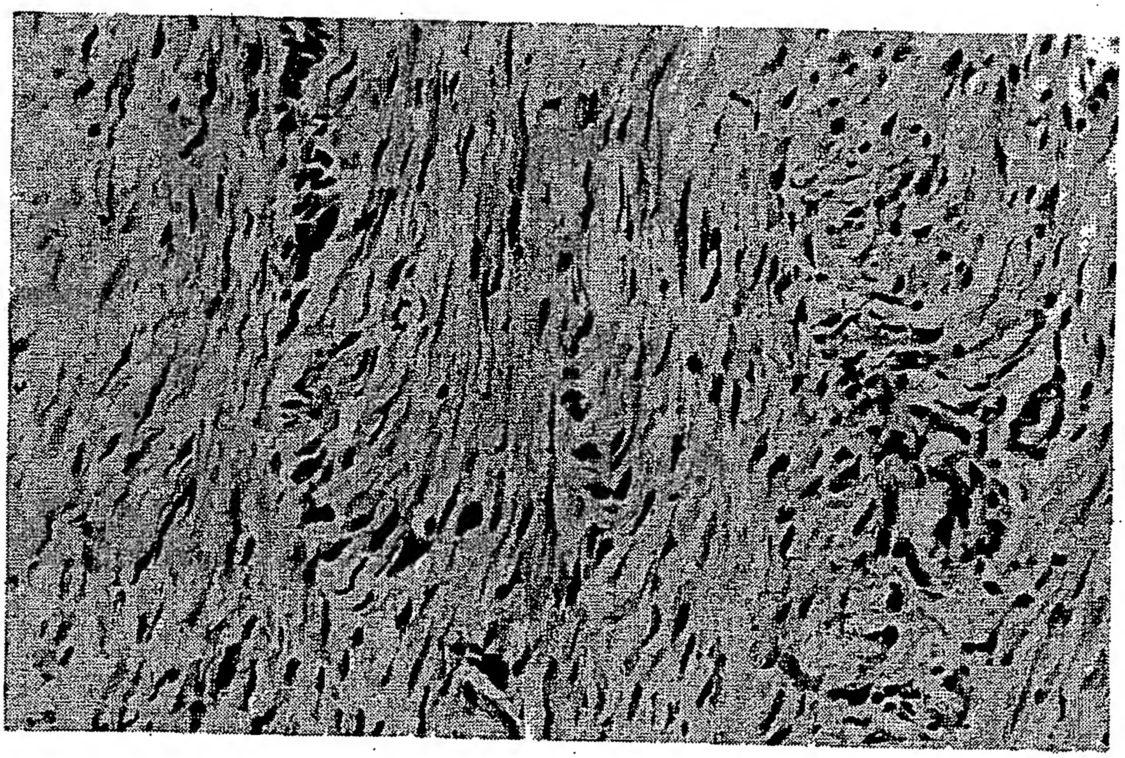


FIG. 4

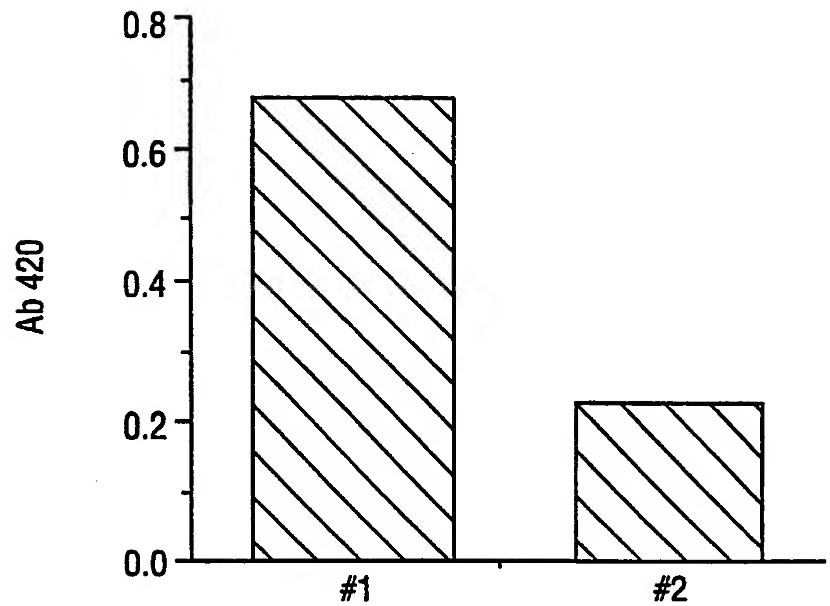

0.8
0.6
0.4
0.2
0.0
Ab 420
#1
#2

FIG. 5A

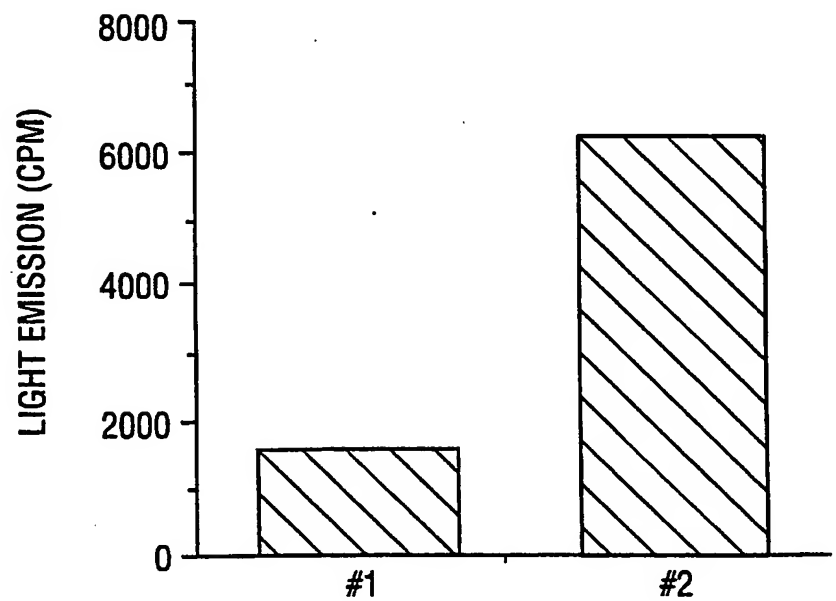

8000
6000
4000
2000
0
LIGHT EMISSION (CPM)
#1
#2

FIG. 5B

FIG. 6A

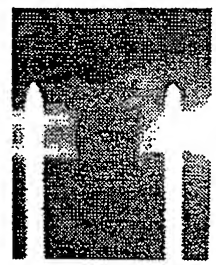


FIG. 6B

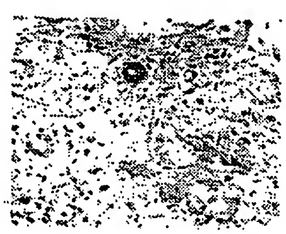


FIG. 6C

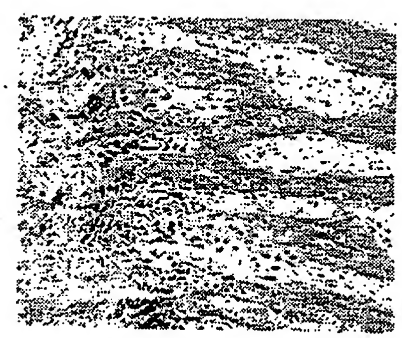


FIG. 6D

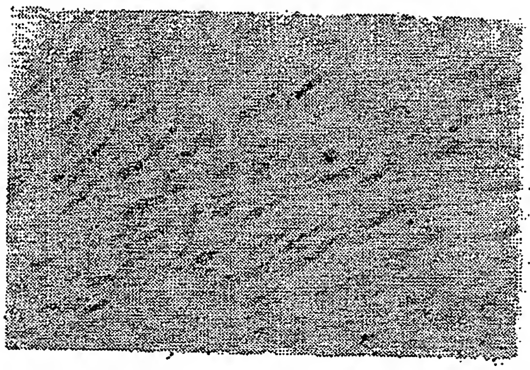


FIG. 7A

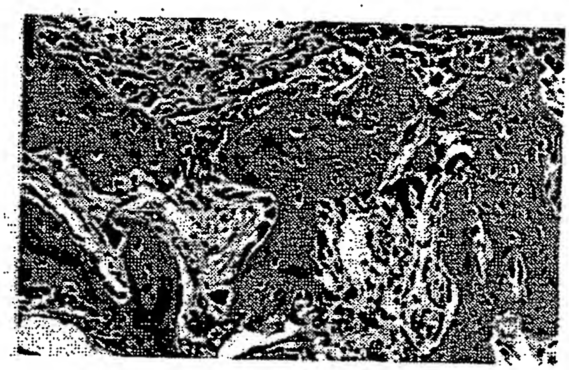


FIG. 7B

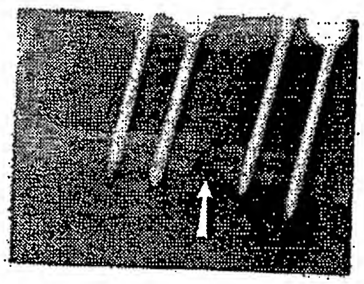


FIG. 8A

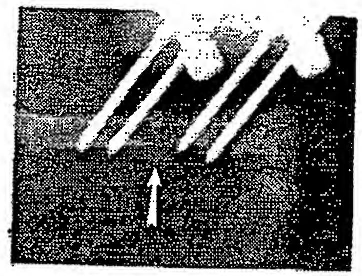


FIG. 8B


FIG. 8C

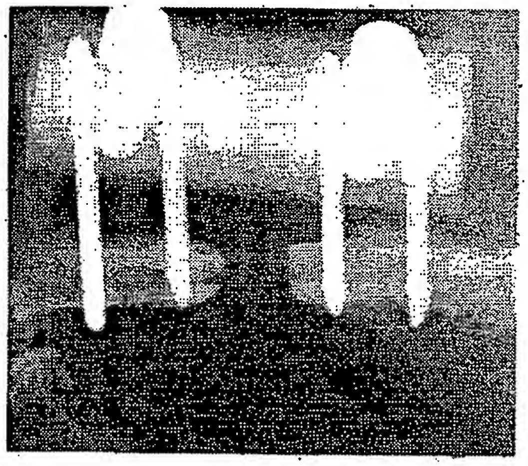


FIG. 9A

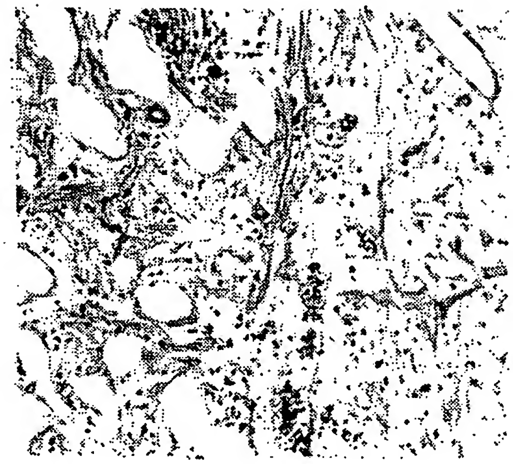
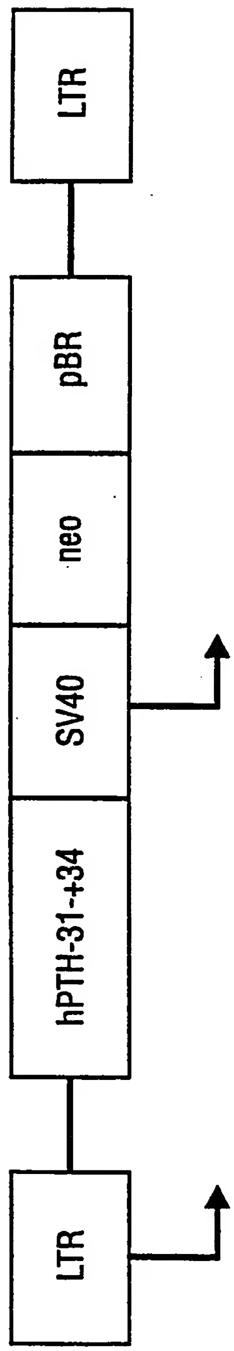


FIG. 9B



PLJ-hPTH 1-34

FIG. 10

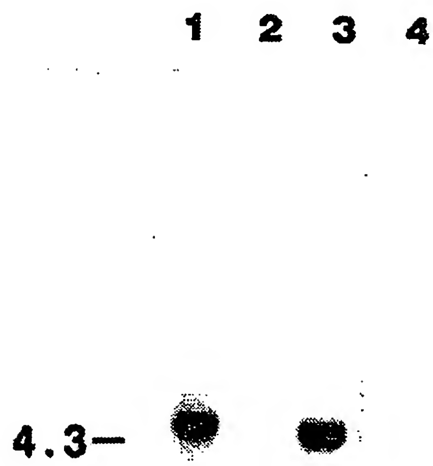


FIG. 11

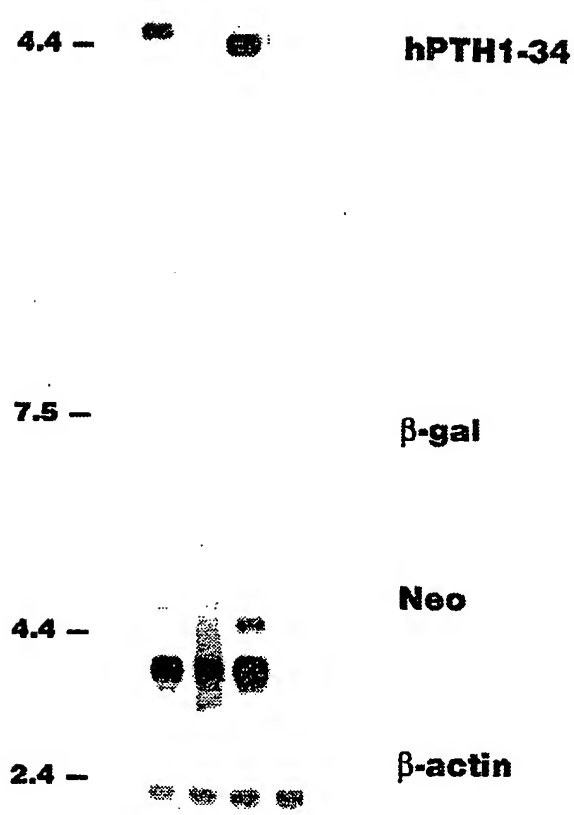


FIG. 12

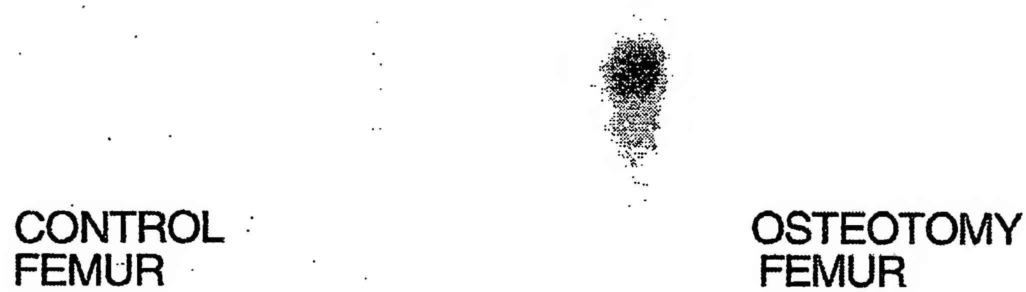
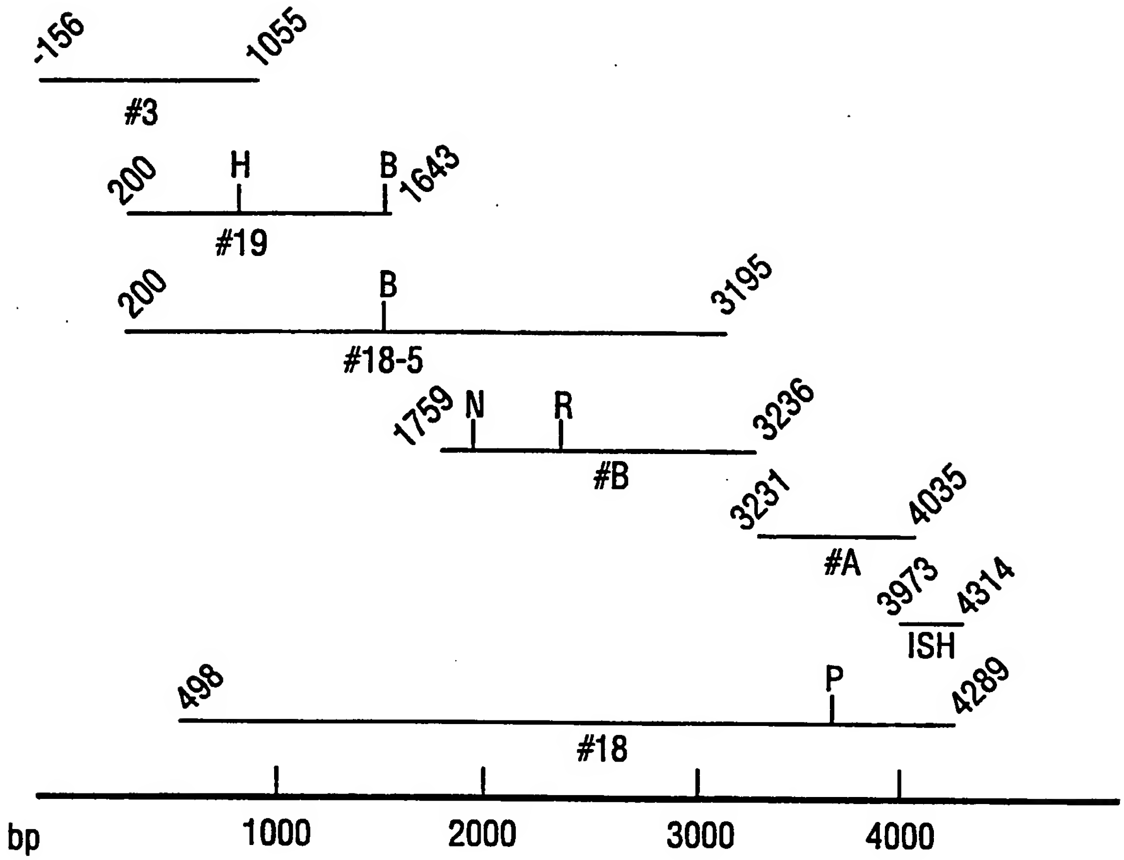


FIG. 13



-156
1055
#3
200
H
B
1643
#19
200
B
3195
#18-5
1759
N
R
3236
#B
3231
4035
#A
3973
4314
ISH
498
P
4289
#18
bp
1000
2000
3000
4000

FIG. 14

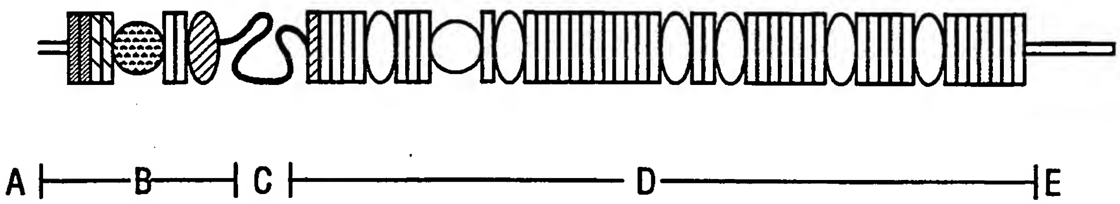


FIG. 15A

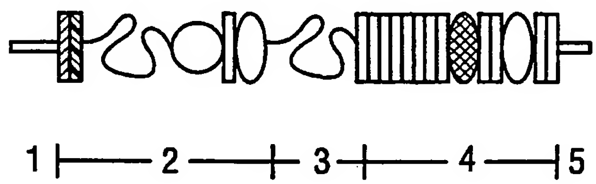


FIG. 15B

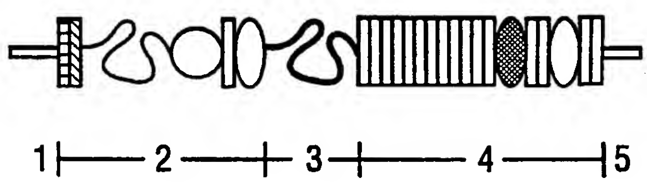
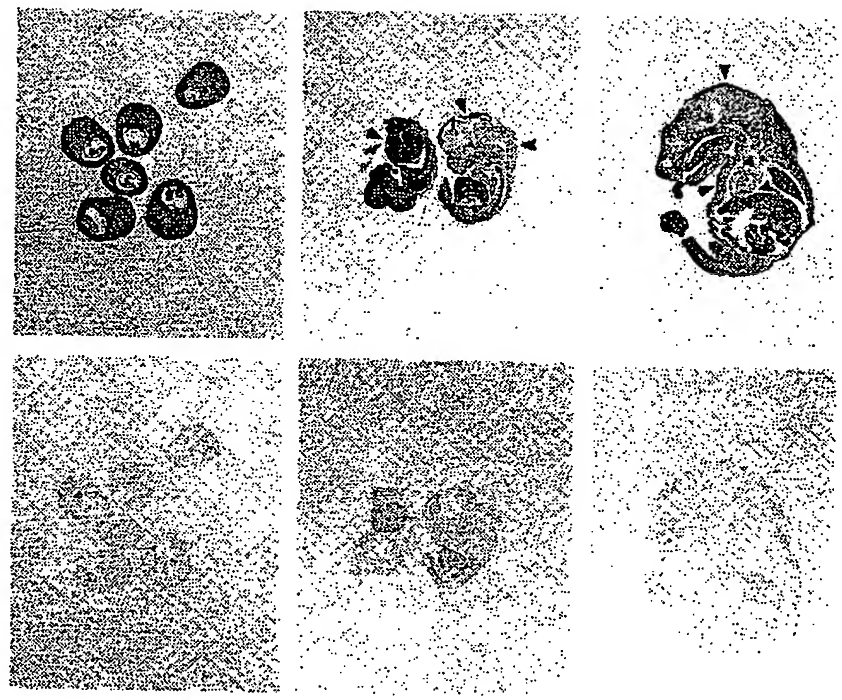


FIG. 15C

anti-sense probe

sense probe



day 8.5 - 9.0 day 13.5 day 16.5

FIG. 16

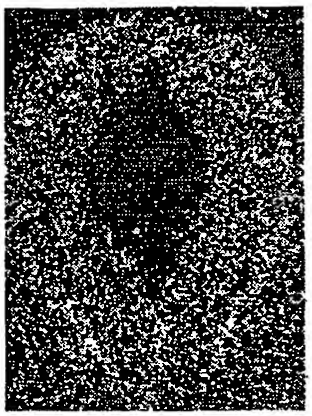


FIG. 17A

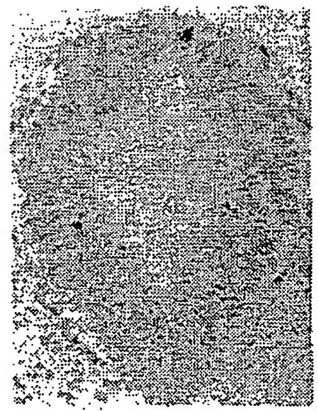


FIG. 17B

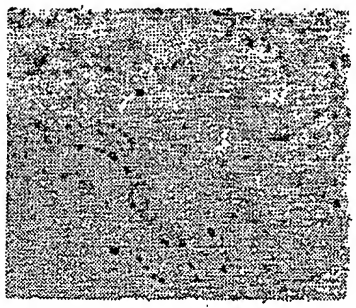


FIG. 17C

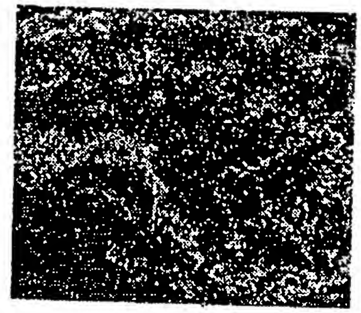


FIG. 17D

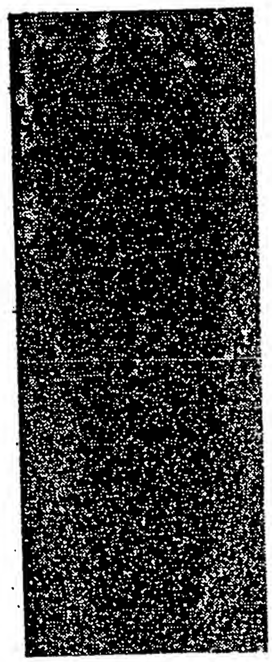


FIG. 18A

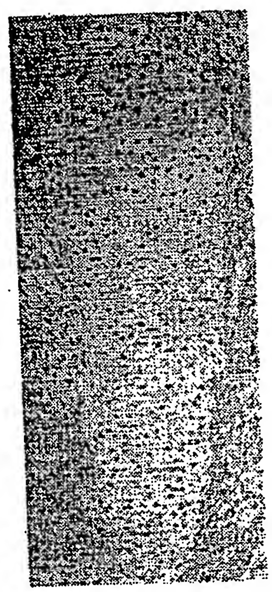


FIG. 18B

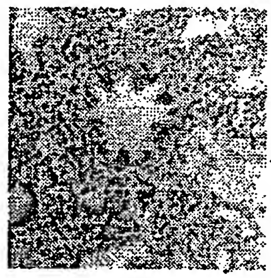


FIG. 18C

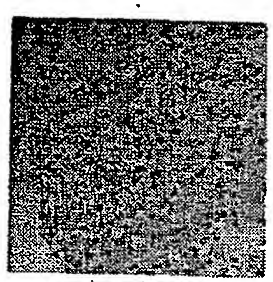


FIG. 18D

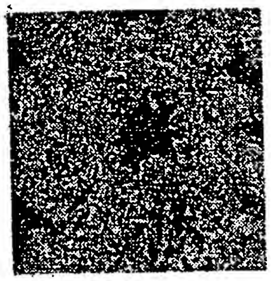


FIG. 18E

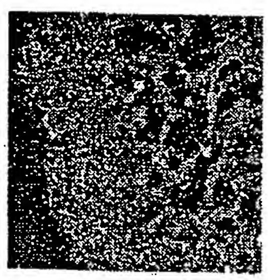


FIG. 18F

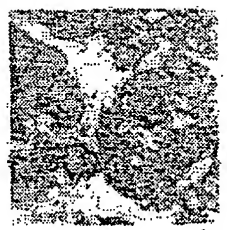


FIG. 18G

FIG. 18H

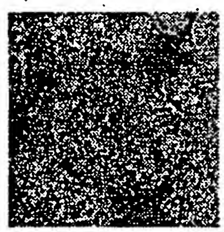


FIG. 18I

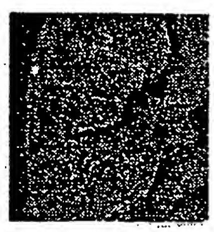


FIG. 18J

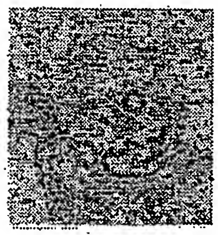


FIG. 18K

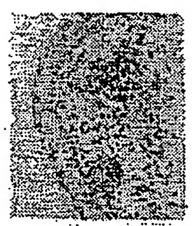


FIG. 18L

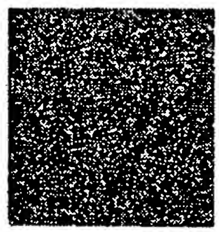


FIG. 18M

FIG. 18N

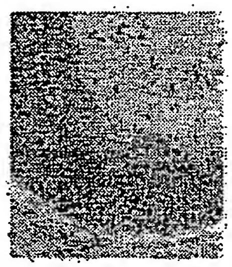


FIG. 180

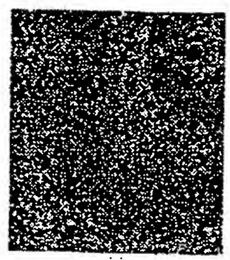


FIG. 18P

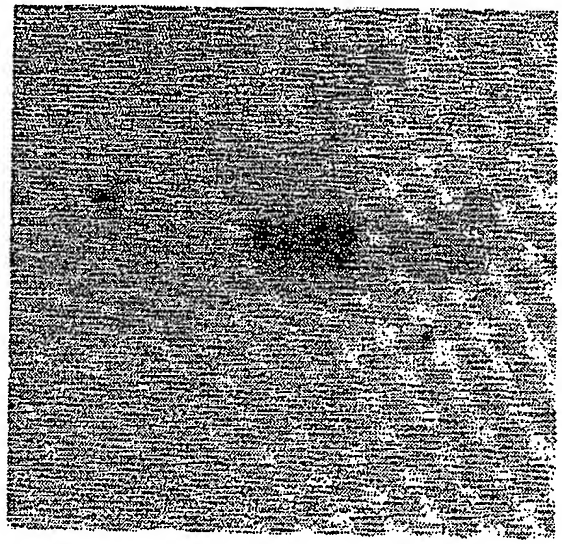


FIG. 19

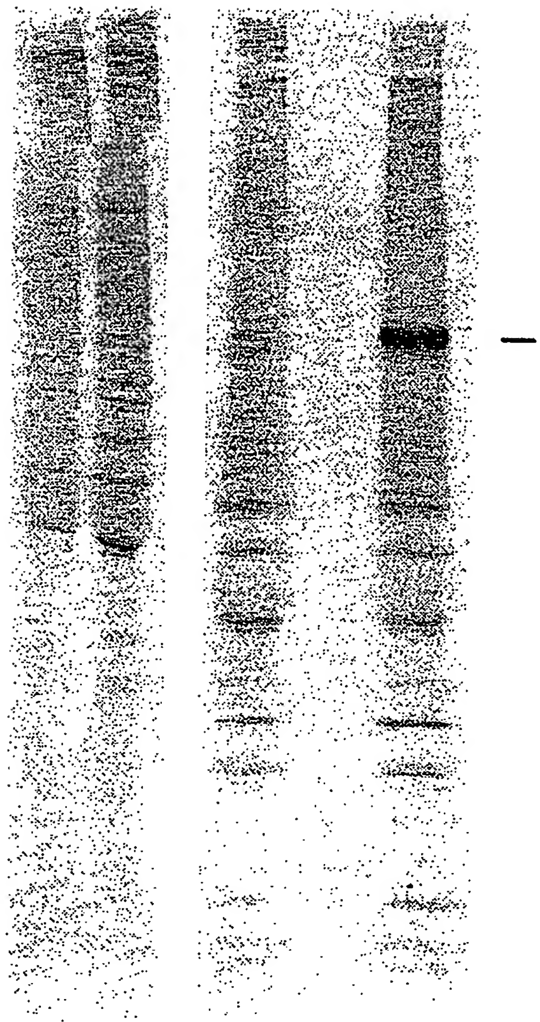


FIG. 20

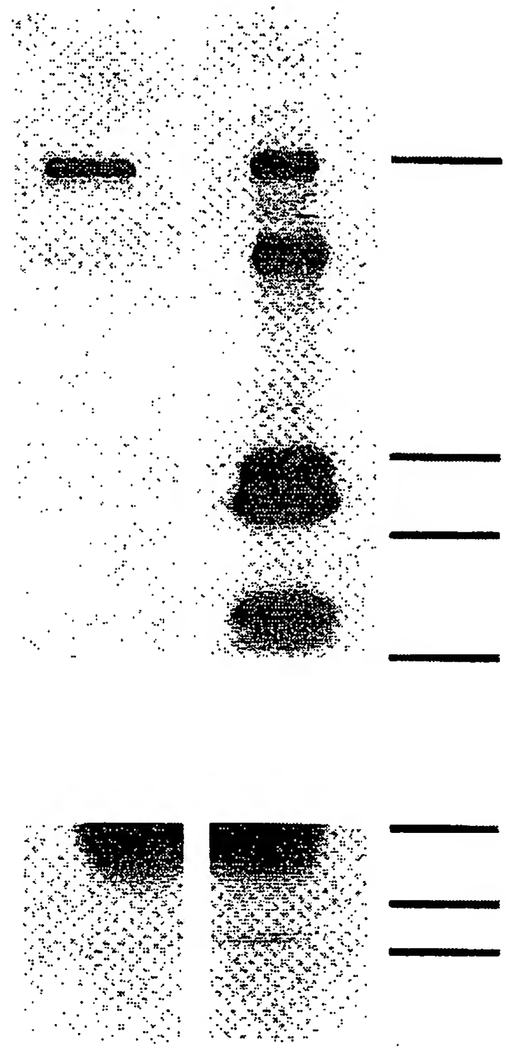


FIG. 21

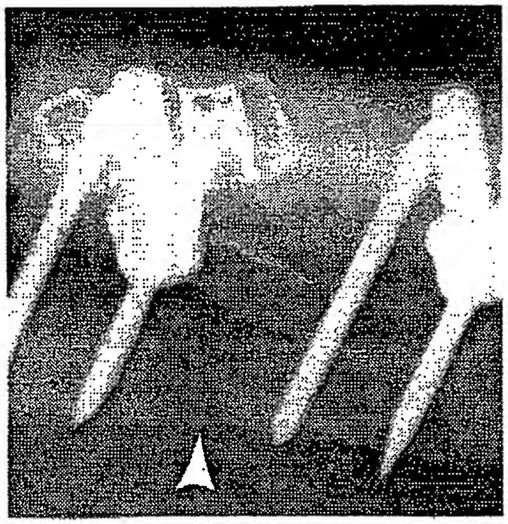


FIG. 22A

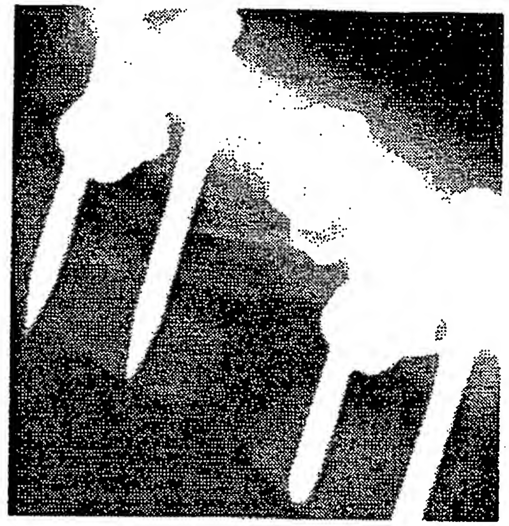


FIG. 22B

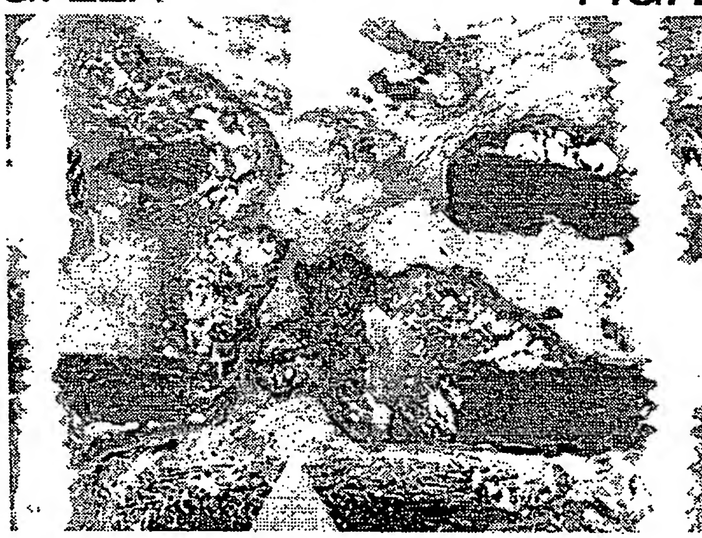


FIG. 22C

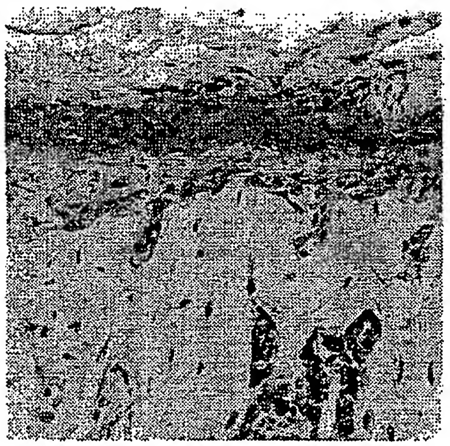


FIG. 23A

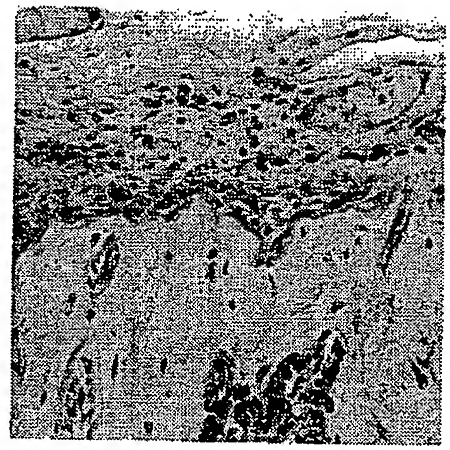


FIG. 23B

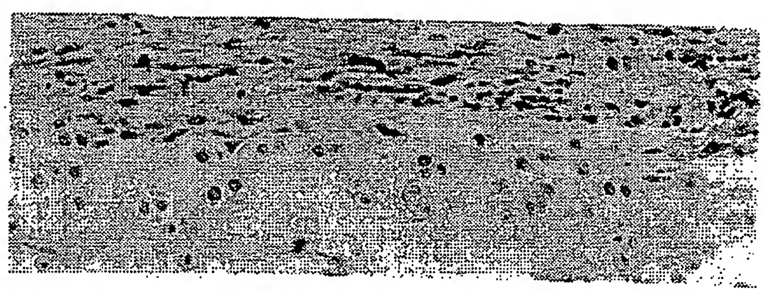


FIG. 23C

```
MIPGNRMLMV VLLCQVLLGG ATDASLMPET GKKKVAEIQG HAGGRRSGQS HELLRDFEAT LLQMFGLRRR
PQPSKSAVIP DYMSDLYRLQ SGEEEEEEQS QGTGLEYPER PASSANTVSS FHHEEHLENI PGTSESSAFR
FFFNLSSIPE NEVISSAELR LFREQVDQGP DWEQGFHRMN IYEVMKPPAE MVPGHLITRL LDTSLVRHNV
TRWETFDVSP AVLRWTREKQ PNYGLAIEVT HLHQTRTHQG QHVSISRSLP QGSGNWAQLR PLLVTFGHDG
RGHTLTRRSA KRSPKHHPQR SSKKNKNCRR HSLYVDFSDV GWNDWIVAPP GYQAFYCHGD CPFPLADHLN
STNHAIVQTL VNSVNSSIPK ACCVPTELSA ISMLYLDEYD KVVLKNYQEM VVEGCGCRYP YDVPDYA
```

FIG. 24

ATG CGC CAG GCC GCA TTG GGG CTG CTG GCA CTA CTC CTG CTG GCG CTG CTG GGC 54
M R Q A A L G L L A L L L L A L L G 18

CCC GGC GGC CGA GGG GTG GGC CGG CCG GGC AGC GGG GCA CAG GCG GGG GCG GGG 108
P G G R G V G R P G S G A Q A G A G 36

CGC TGG GCC CAA CGC TTC AAG GTG GTC TTT GCG CCT GTG ATC TGC AAG CGG ACC 162
R W A Q R F K V V F A P V I C K R T 54

TGT CTG AAG GGC CAG TGT CGG GAC AGC TGT CAG CAG GGC TCC AAC ATG ACG CTC 216
C L K G Q C R D S C Q Q G S N M T L 72

ATC GGA GAG AAC GGC CAC AGC ACC GAC ACG CTC ACC GGT TCT GCC TTC CGC GTG 270
I G E N G H S T D T L T G S A F R V 90

GTG GTG TGC CCT CTA CCC TGC ATG AAC GGT GGC CAG TGC TCT TCC CGA AAC CAG 324
V V C P L P C M N G G Q C S S R N Q 108

TGC CTG TGT CCC CCG GAT TTC ACG GGG CGC TTC TGC CAG GTG CCT GCT GCA GGA 378
C L C P P D F T G R F C Q V P A A G 126

ACC GGA GCT GGC ACC GGG AGT TCA GGC CCC GGC TGG CCC GAC CGG GCC ATG TCC 432
T G A G T G S S G P G W P D R A M S 144

ACA GGC CCG CTG CCG CCC CTT GCC CCA GAA GGA GAG TCT GTG GCT AGC AAA CAC 486
T G P L P P L A P E G E S V A S K H 162

GCC ATT TAC GCG GTG CAG GTG ATC GCA GAT CCT CCC GGG CCG GGG GAG GGT CCT 540
A I Y A V Q V I A D P P G P G E G P 180

CCT GCA CAA CAT GCA GCC TTC TTG GTG CCC CTG GGG CCA GGA CAA ATC TCG GCA 594
P A Q H A A F L V P L G P G Q I S A 198

GAA GTG CAG GCT CCG CCC CCC GTG GTG AAC GTG CGT GTC CAT CAC CCT CCT GAA 648
E V Q A P P P V V N V R V H H P P E 216

GCT TCC GTT CAG GTG CAC CGC ATC GAG GGG CCG AAC GCT GAA GGC CCA GCC TCT 702
A S V Q V H R I E G P N A E G P A S 234

TCC CAG CAC TTG CTG CCG CAT CCC AAG CCC CAG CAC CCG AGG CCA CCC ACT CAA 756
S Q H L L P H P K P Q H P R P P T Q 252

AAG CCA CTG GGC CGC TGC TTC CAG GAC ACA TTG CCC AAG CAG CCT TGT GGC AGC 810
K P L G R C F Q D T L P K Q P C G S 270

AAC CCT TTG CCT GGC CTT ACC AAG CAG GAA GAT TGC TGC GGT AGC ATC GGT ACT 864
N P L P G L T K Q E D C C G S I G T 288

GCC TGG GGA CAA AGC AAG TGT CAC AAG TGC CCA CAG CTT CAG TAT ACA GGG GTG 918
A W G Q S K C H K C P Q L Q Y T G V 306

CAG AAG CCT GTA CCT GTA CGT GGG GAG GTG GGT GCT GAC TGC CCC CAG GGC TAC 972
Q K P V P V R G E V G A D C P Q G Y 324

AAG AGG CTC AAC AGC ACC CAC TGC CAG GAT ATC AAC GAA TGT GCG ATG CCC GGG 1026
K R L N S T H C Q D I N E C A M P G 342

FIG. 25-1

```
AAT GTG TGC CAT GGT GAC TGC CTC AAC AAC CCT GGC TCT TAT CGC TGT GTC TGC 1080
 N   V   C   H   G   D   C   L   N   N   P   G   S   Y   R   C   V   C  360

CCG CCC GGT CAT AGC TTG GGT CCC CTC GCA GCA CAG TGC ATT GCC GAC AAA CCA 1134
 P   P   G   H   S   L   G   P   L   A   A   Q   C   I   A   D   K   P  378

GAG GAG AAG AGC CTG TGT TTC CGC CTT GTG AGC ACC GAA CAC CAG TGC CAG CAC 1188
 E   E   K   S   L   C   F   R   L   V   S   T   E   H   Q   C   Q   H  396

CCT CTG ACC ACA CGC CTA ACC CGC CAG CTC TGC TGC TGT AGT GTG GGT AAA GCC 1242
 P   L   T   T   R   L   T   R   Q   L   C   C   C   S   V   G   K   A  414

TGG GGT GCC CGG TGC CAG CGC TGC CCG GCA GAT GGT ACA GCA GCC TTC AAG GAG 1296
 W   G   A   R   C   Q   R   C   P   A   D   G   T   A   A   F   K   E  432

ATC TGC CCC GGC TGG GAA AGG GTA CCA TAT CCT CAC CTC CCA CCA GAC GCT CAC 1350
 I   C   P   G   W   E   R   V   P   Y   P   H   L   P   P   D   A   H  450

CAT CCA GGG GGA AAG CGA CTT CTC CCT CTT CCT GCA CCC GAC GGG CCA CCC AAA 1404
 H   P   G   G   K   R   L   L   P   L   P   A   P   D   G   P   P   K  468

CCC CAG CAG CTT CCT GAA AGC CCC AGC CGA GCA CCA CCC CTC GAG GAC ACA GAG 1458
 P   Q   Q   L   P   E   S   P   S   R   A   P   P   L   E   D   T   E  486

GAA GAG AGA GGA GTG ACC ATG GAT CCA CCA GTG AGT GAG GAG CGA TCG GTG CAG 1512
 E   E   R   G   V   T   M   D   P   P   V   S   E   E   R   S   V   Q  504

CAG AGC CAC CCC ACT ACC ACC ACC TCA CCC CCC CGG CCT TAC CCA GAG CTC ATC 1566
 Q   S   H   P   T   T   T   T   S   P   P   R   P   Y   P   E   L   I  522

TCT CGC CCC TCC CCA CCT ACC TTC CAC CGG TTC CTG CCA GAC TTG CCC CCA TCC 1620
 S   R   P   S   P   P   T   F   H   R   F   L   P   D   L   P   P   S  540

CGA AGT GCA GTG GAG ATC GCC CCC ACT CAG GTC ACA GAG ACC GAT GAG TGC CGA 1674
 R   S   A   V   E   I   A   P   T   Q   V   T   E   T   D   E   C   R  558

TTG AAC CAG AAT ATC TGT GGC CAT GGA CAG TGT GTG CCT GGC CCC TCG GAT TAC 1728
 L   N   Q   N   I   C   G   H   G   Q   C   V   P   G   P   S   D   Y  576

TCC TGC CAC TGC AAC GCT GGC TAC CGG TCA CAC CCG CAG CAC CGC TAC TGT GTT 1782
 S   C   H   C   N   A   G   Y   R   S   H   P   Q   H   R   Y   C   V  594

GAT GTG AAC GAG TGC GAG GCA GAG CCC TGC GGC CCC GGG AAA GGC ATC TGT ATG 1836
 D   V   N   E   C   E   A   E   P   C   G   P   G   K   G   I   C   M  612

AAC ACT GGT GGC TCC TAC AAT TGT CAC TGC AAC CGA GGC TAC CGC CTC CAC GTG 1890
 N   T   G   G   S   Y   N   C   H   C   N   R   G   Y   R   L   H   V  630

GGT GCA GGG GGC CGC TCG TGC GTG GAC CTG AAC GAG TGC GCC AAG CCT CAC CTG 1944
 G   A   G   G   R   S   C   V   D   L   N   E   C   A   K   P   H   L  648

TGT GGG GAC GGT GGC TTC TGC ATC AAC TTC CCT GGT CAC TAC AAA TGC AAC TGC 1998
 C   G   D   G   G   F   C   I   N   F   P   G   H   Y   K   C   N   C  666

TAT CCT GGC TAC CGG CTC AAG GCC TCC CGA CCG CCC ATT TGC GAA GAC ATC GAC 2052
 Y   P   G   Y   R   L   K   A   S   R   P   P   I   C   E   D   I   D  684

GAG TGT CGC GAC CCT AGC ACC TGC CCT GAT GGC AAA TGT GAA AAC AAA CCT GGC 2106
 E   C   R   D   P   S   T   C   P   D   G   K   C   E   N   K   P   G  702
```

FIG. 25-2

AGC TTC AAG TGC ATC GCC TGC CAG CCT GGC TAC CGT AGC CAG GGG GGC GGG GCC 2160
S F K C I A C Q P G Y R S Q G G G A 720

TGT CGT GAT GTC AAC GAA TGC TCC GAA GGT ACC CCC TGC TCT CCT GGA TGG TGT 2214
C R D V N E C S E G T P C S P G W C 738

GAG AAA CTT CCG GGT TCT TAC CGT TGC ACG TGT GCC CAG GGG ATA CGA ACC CGC 2268
E K L P G S Y R C T C A Q G I R T R 756

ACA GGA CGC CTC AGT TGC ATA GAC GTG GAT GAC TGT GAG GCT GGG AAA GTG TGC 2322
T G R L S C I D V D D C E A G K V C 774

CAA GAT GGC ATC TGC ACG AAC ACA CCA GGC TCT TTC CAG TGT CAG TGC CTC TCC 2376
Q D G I C T N T P G S F Q C Q C L S 792

GGC TAT CAT CTG TCA AGG GAT CGG AGC CGC TGT GAG GAC ATT GAT GAA TGT GAC 2430
G Y H L S R D R S R C E D I D E C D 810

TTC CCT GCG GCC TGC ATC GGG GGT GAC TGC ATC AAT ACC AAT GGT TCC TAC AGA 2484
F P A A C I G G D C I N T N G S Y R 828

TGT CTC TGT CCC CTG GGT CAT CGG TTG GTG GGC GGC AGG AAG TGC AAG AAA GAT 2538
C L C P L G H R L V G G R K C K K D 846

ATA GAT GAG TGC AGC CAG GAC CCA GGC CTG TGC CTG CCC CAT GCC TGC GAG AAC 2592
I D E C S Q D P G L C L P H A C E N 864

CTC CAG GGC TCC TAT GTC TGT GTC TGT GAT GAG GGT TTC ACA CTC ACC CAG GAC 2646
L Q G S Y V C V C D E G F T L T Q D 882

CAG CAT GGG TGT GAG GAG GTG GAG CAG CCC CAC CAC AAG AAG GAG TGC TAC CTT 2700
Q H G C E E V E Q P H H K K E C Y L 900

AAC TTC GAT GAC ACA GTG TTC TGT GAC AGC GTA TTG GCT ACC AAT GTC ACT CAG 2754
N F D D T V F C D S V L A T N V T Q 918

CAG GAA TGC TGT TGC TCT CTG GGA GCT GGC TGG GGA GAC CAC TGC GAA ATC TAT 2808
Q E C C C S L G A G W G D H C E I Y 936

CCC TGT CCA GTC TAC AGC TCA GCC GAA TTT CAC AGC CTG GTG CCT GAT GGG AAA 2862
P C P V Y S S A E F H S L V P D G K 954

AGG CTA CAC TCA GGA CAA CAA CAT TGT GAA CTA TGC ATT CCT GCC CAC CGT GAC 2916
R L H S G Q Q H C E L C I P A H R D 972

ATC GAC GAA TGC ATA TTG TTT GGG GCA GAG ATC TGC AAG GAG GGC AAG TGT GTG 2970
I D E C I L F G A E I C K E G K C V 990

AAC TCG CAG CCC GGC TAC GAG TGC TAC TGC AAG CAG GGC TTC TAC TAC GAT GGC 3024
N S Q P G Y E C Y C K Q G F Y Y D G 1008

AAC CTG CTG GAG TGC GTG GAC GTG GAC GAG TGC TTG GAT GAG TCT AAC TGC AGG 3078
N L L E C V D V D E C L D E S N C R 1026

AAC GGA GTG TGT GAG AAC ACG TGG CGG CTA CCG TGT GCC TGC ACT CCG CCG GCA 3132
N G V C E N T W R L P C A C T P P A 1044

GAG TAC AGT CCC GCA CAG GCC CAG TGT CTG AGC CCG GAG GAG ATG GAG CAC GCC 3186
E Y S P A Q A Q C L S P E E M E H A 1062

FIG. 25-3

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CCA	GAG	AGA	CGT	GAA	GTG	TGC	TGG	GGC	CAG	CGA	GGA	GAG	GAC	GGC	ATG	TGT	ATG	3240
P	E	R	R	E	V	C	W	G	Q	R	G	E	D	G	M	C	M	1080
GGG	CCC	CTG	GCG	GGA	CCT	GCC	CTC	ACT	TTT	GAT	GAC	TGC	TGC	TGC	CGC	CAG	CCG	3294
G	P	L	A	G	P	A	L	T	F	D	D	C	C	C	R	Q	P	1098
CGG	CTG	GGG	TAC	CAG	TGC	AGA	CCG	TGC	CCG	CCA	CGT	GGC	ACC	GGG	TCC	CAG	TGC	3348
R	L	G	Y	Q	C	R	P	C	P	P	R	G	T	G	S	Q	C	1116
CCG	ACT	TCA	CAG	AGT	GAG	AGC	AAT	TCT	TTC	TGG	GAC	ACA	AGC	CCC	CTG	CTA	CTG	3402
P	T	S	Q	S	E	S	N	S	F	W	D	T	S	P	L	L	L	1134
GGG	AAG	TCT	CCG	CGA	GAC	GAA	GAC	AGC	TCA	GAG	GAG	GAT	TCA	GAT	GAG	TGC	CGT	3456
G	K	S	P	R	D	E	D	S	S	E	E	D	S	D	E	C	R	1152
TGT	GTG	AGC	GGA	CCG	TGT	GTG	CCA	CGG	CCA	GGC	GGG	GCG	GTA	TGC	GAG	TGT	CCT	3510
C	V	S	G	P	C	V	P	R	P	G	G	A	V	C	E	C	P	1170
GGA	GGC	TTT	CAG	CTG	GAC	GCC	TCC	CGT	GCC	CGC	TGC	GTG	GAC	ATT	GAT	GAG	TGC	3564
G	G	F	Q	L	D	A	S	R	A	R	C	V	D	I	D	E	C	1188
CGA	GAA	CTG	AAC	CAG	CGG	GGA	CTG	CTG	TGT	AAG	AGC	GAG	CGG	TGC	GTG	AAC	ACC	3618
R	E	L	N	Q	R	G	L	L	C	K	S	E	R	C	V	N	T	1206
AGT	GGA	TCC	TTC	CGC	TGT	GTC	TGC	AAA	GCT	GGC	TTC	ACG	CGC	AGC	CGC	CCT	CAC	3672
S	G	S	F	R	C	V	C	K	A	G	F	T	R	S	R	P	H	1224
GGG	CCT	GCG	TGC	CTC	AGC	GCC	GCC	GCT	GAT	GAT	GCA	GCC	ATA	GCC	CAC	ACC	TCA	3726
G	P	A	C	L	S	A	A	A	D	D	A	A	I	A	H	T	S	1242
GTG	ATC	GAT	CAT	CGA	GGG	TAT	TTT	CAC	TGA									
V	I	D	H	R	G	Y	F	H	*									

FIG. 25-4

Met Arg Gln Ala Ala Leu Gly Leu Leu Ala Leu Leu Leu Leu Ala Leu Leu Gly Pro Gly Gly Arg 22
Gly Val Gly Arg Pro Gly Ser Gly Ala Gln Ala Gly Ala Gly Arg Trp Ala Gln Arg Phe Lys Val 44
Val Phe Ala Pro Val Ile Cys Lys Arg Thr Cys Leu Lys Gly Gln Cys Arg Asp Ser Cys Gln Gln 66
Gly Ser Asn Met Thr Leu Ile Gly Glu Asn Gly His Ser Thr Asp Thr Leu Thr Gly Ser Ala Phe 88
Arg Val Val Val Cys Pro Leu Pro Cys Met Asn Gly Gly Gln Cys Ser Ser Arg Asn Gln Cys Leu 110
Cys Pro Pro Asp Phe Thr Gly Arg Phe Cys Gln Val Pro Ala Ala Gly Thr Gly Ala Gly Thr Gly 132
Ser Ser Gly Pro Gly Trp Pro Asp Arg Ala Met Ser Thr Gly Pro Leu Pro Pro Leu Ala Pro Glu 154
Gly Glu Ser Val Ala Ser Lys His Ala Ile Tyr Ala Val Gln Val Ile Ala Asp Pro Pro Gly Pro 176
Gly Glu Gly Pro Pro Ala Gln His Ala Ala Phe Leu Val Pro Leu Gly Pro Gly Gln Ile Ser Ala 198
Glu Val Gln Ala Pro Pro Pro Val Val Asn Val Arg Val His His Pro Pro Glu Ala Ser Val Gln 220
Val His Arg Ile Glu Gly Pro Asn Ala Glu Gly Pro Ala Ser Ser Gln His Leu Leu Pro His Pro 242
Lys Pro Pro His Pro Arg Pro Pro Thr Gln Lys Pro Leu Gly Arg Cys Phe Gln Asp Thr Leu Pro 264
Lys Gln Pro Cys Gly Ser Asn Pro Leu Pro Gly Leu Thr Lys Gln Glu Asp Cys Cys Gly Ser Ile 286
Gly Thr Ala Trp Gly Gln Ser Lys Cys His Lys Cys Pro Gln Leu Gln Tyr Thr Gly Val Gln Lys 308
Pro Val Pro Val Arg Gly Glu Val Gly Ala Asp Cys Pro Gln Gly Tyr Lys Arg Leu Asn Ser Thr 330
His Cys Gln Asp Ile Asn Glu Cys Ala Met Pro Gly Asn Val Cys His Gly Asp Cys Leu Asn Asn 352
Pro Gly Ser Tyr Arg Cys Val Cys Pro Pro Gly His Ser Leu Gly Pro Leu Ala Ala Gln Cys Ile 374
Ala Asp Lys Pro Glu Glu Lys Ser Leu Cys Phe Arg Leu Val Ser Thr Glu His Gln Cys Gln His 396
Pro Leu Thr Thr Arg Leu Thr Arg Gln Leu Cys Cys Cys Ser Val Gly Lys Ala Trp Gly Ala Arg 418
Cys Gln Arg Cys Pro Ala Asp Gly Thr Ala Ala Phe Lys Glu Ile Cys Pro Gly Trp Glu Arg Val 440
Pro Tyr Pro His Leu Pro Pro Asp Ala His His Pro Gly Gly Lys Arg Leu Leu Pro Leu Pro Ala 462
Pro Asp Gly Pro Pro Lys Pro Gln Gln Leu Pro Glu Ser Pro Ser Arg Ala Pro Pro Leu Glu Asp 484

FIG. 26-1

Thr Glu Glu Glu Arg Gly Val Thr Met Asp Pro Pro Val Ser Glu Glu Arg Ser Val Gln Gln Ser 506
His Pro Thr Thr Thr Thr Ser Pro Pro Arg Pro Tyr Pro Glu Leu Ile Ser Arg Pro Ser Pro Pro 528
Thr Phe His Arg Phe Leu Pro Asp Leu Pro Pro Ser Arg Ser Ala Val Glu Ile Ala Pro Thr Gln 550
Val Thr Glu Thr Asp Glu Cys Arg Leu Asn Gln Asn Ile Cys Gly His Gly Gln Cys Val Pro Gly 572
Pro Ser Asp Tyr Ser Cys His Cys Asn Ala Gly Tyr Arg Ser His Pro Gln His Arg Tyr Cys Val 594
Asp Val Asn Glu Cys Glu Ala Glu Pro Cys Gly Pro Gly Lys Gly Ile Cys Met Asn Thr Gly Gly 616
Ser Tyr Asn Cys His Cys Asn Arg Gly Tyr Arg Leu His Val Gly Ala Gly Gly Arg Ser Cys Val 638
Asp Leu Asn Glu Cys Ala Lys Pro His Leu Cys Gly Asp Gly Gly Phe Cys Ile Asn Phe Pro Gly 660
His Tyr Lys Cys Asn Cys Tyr Pro Gly Tyr Arg Leu Lys Ala Ser Arg Pro Pro Ile Cys Glu Asp 682
Ile Asp Glu Cys Arg Asp Pro Ser Thr Cys Pro Asp Gly Lys Cys Glu Asn Lys Pro Gly Ser Phe 704
Lys Cys Ile Ala Cys Gln Pro Gly Tyr Arg Ser Gln Gly Gly Gly Ala Cys Arg Asp Val Asn Glu 726
Cys Ser Glu Gly Thr Pro Cys Ser Pro Gly Trp Cys Glu Lys Leu Pro Gly Ser Tyr Arg Cys Thr 748
Cys Ala Gln Gly Ile Arg Thr Arg Thr Gly Arg Leu Ser Cys Ile Asp Val Asp Asp Cys Glu Ala 770
Gly Lys Val Cys Gln Asp Gly Ile Cys Thr Asn Thr Pro Gly Ser Phe Gln Cys Gln Cys Leu Ser 792
Gly Tyr His Leu Ser Arg Asp Arg Ser Arg Cys Glu Asp Ile Asp Glu Cys Asp Phe Pro Ala Ala 814
Cys Ile Gly Gly Asp Cys Ile Asn Thr Asn Gly Ser Tyr Arg Cys Leu Cys Pro Leu Gly His Arg 836
Leu Val Gly Gly Arg Lys Cys Lys Lys Asp Ile Asp Glu Cys Ser Gln Asp Pro Gly Leu Cys Leu 858
Pro His Ala Cys Glu Asn Leu Gln Gly Ser Tyr Val Cys Val Cys Asp Glu Gly Phe Thr Leu Thr 880
Gln Asp Gln His Gly Cys Glu Glu Val Glu Gln Pro His His Lys Lys Glu Cys Tyr Leu Asn Phe 902
Asp Asp Thr Val Phe Cys Asp Ser Val Leu Ala Thr Asn Val Thr Gln Gln Glu Cys Cys Cys Ser 924
Leu Gly Ala Gly Trp Gly Asp His Cys Glu Ile Tyr Pro Cys Pro Val Tyr Ser Ser Ala Glu Phe 946
His Ser Leu Val Pro Asp Gly Lys Arg Leu His Ser Gly Gln Gln His Cys Glu Leu Cys Ile Pro 968

FIG. 26-2

Ala His Arg Asp Ile Asp Glu Cys Ile Leu Phe Gly Ala Glu Ile Cys Lys Glu Gly Lys Cys Val 990
Asn Ser Gln Pro Gly Tyr Glu Cys Tyr Cys Lys Gln Gly Phe Tyr Tyr Asp Gly Asn Leu Leu Glu 1012
Cys Val Asp Val Asp Glu Cys Leu Asp Glu Ser Asn Cys Arg Asn Gly Val Cys Glu Asn Thr Trp 1034
Arg Leu Pro Cys Ala Cys Thr Pro Pro Ala Glu Tyr Ser Pro Ala Gln Ala Gln Cys Leu Ile Pro 1056
Glu Arg Trp Ser Thr Pro Gln Arg Asp Val Lys Cys Ala Gly Ala Ser Glu Glu Arg Thr Ala Cys 1078
Val Trp Gly Pro Trp Ala Gly Pro Ala Leu Thr Phe Asp Asp Cys Cys Cys Arg Gln Pro Arg Leu 1100
Gly Thr Gln Cys Arg Pro Cys Pro Pro Arg Gly Thr Gly Ser Gln Cys Pro Thr Ser Gln Ser Glu 1122
Ser Asn Ser Phe Trp Asp Thr Ser Pro Leu Leu Leu Gly Lys Ser Pro Arg Asp Glu Asp Ser Ser 1144
Glu Glu Asp Ser Asp Glu Cys Arg Cys Val Ser Gly Pro Cys Val Pro Arg Pro Gly Gly Ala Val 1166
Cys Glu Cys Pro Gly Gly Phe Gln Leu Asp Ala Ser Arg Ala Arg Cys Val Asp Ile Asp Glu Cys 1188
Arg Glu Leu Asn Gln Arg Gly Leu Leu Cys Lys Ser Glu Arg Cys Val Asn Thr Ser Gly Ser Phe 1210
Arg Cys Val Cys Lys Ala Gly Phe Thr Arg Ser Arg Pro His Gly Pro Ala Cys Leu Ser Ala Ala 1232
Ala Asp Asp Ala Ala Ile Ala His Thr Ser Val Ile Asp His Arg Gly Tyr Phe His 1251

FIG. 26-3

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ATGGAGAGCA CCTCCCCGCG AGGTCTCCGG TGCCCACAGC TCTGCAGCCA CTCTGGCGCC ATGAGAGCGC CGACCACCGC   80
TCGCTGCTCC GGATGCATCC AACGGGTGCG TTGGAGGGGC TTCCTGCCAC TTGTCCTGGC TGTCTTGATG GGGACAAGTC  160
ATGCCCAACG GGATTCCATA GGGAGATACG AACCAGCTAG CAGGGATGCG AATCGGTTGT GGCACCCCGT GGGCAGCCAC  240
CCCGCAGCGG CTGCAGCCAA GGTGTACAGT CTGTTCCGAG AGCCTGACGC GCCGGTCCCC GGCTTGTCGC CCTCTGAGTG  320
GAACCAGCCG GCCCAGGGGA ACCCGGGATG GCTCGCAGAG GCCGAGGCCA GGAGGCCACC TCGAACCCAG CAGCTGCGTC  400
GAGTCCAGCC ACCTGTCCAG ACTCGGAGAA GCCATCCCCG GGGCCAGCAG CAGATAGCAG CCCGGGCTGC ACCTTCTGTC  480
GCGCGCCTGG AAACCCCTCA GCGACCCGCG GCTGCACGGC GAGGGCGGCT CACTGGGAGA AATGTCTGCG GGGGACAGTG  560
CTGCCCAGGA TGGACAACAT CAAACAGCAC CAACCACTGT ATCAAACCTG TGTGTCAGCC TCCCTGTCAG AACCGAGGCT  640
CCTGCAGCAG GCCCCAGGTC TGCATCTGCC GTTCTGGCTT CCGTGGGGCG CGCTGTGAGG AGGTCATCCC TGAGGAGGAA  720
TTTGACCCTC AGAATGCCAG GCCTGTGCCC AGACGCTCAG TGGAGAGAGC ACCCGGTCCT CACAGAAGCA GTGAGGCCAG  800
AGGAAGTCTA GTGACCAGAA TACAGCCGCT GGTACCACCA CCATCACCAC CTCCATCTCG GCGCCTCAGC CAGCCCTGGC  880
CCCTGCAGCA GCACTCAGGG CCGTCCAGGA CAGTTCGTCG GTATCCGGCC ACTGGTGCCA ATGGCCAGCT GATGTCCAAC  960
GCTTTGCCTT CAGGACTCGA GCTGAGAGAC AGCAGCCCAC AGGCAGCACA TGTGAACCAT CTCTCACCCC CCTGGGGGCT 1040
GAACCTCACC GAGAAAATCA AGAAAATCAA AGTCGTCTTC ACCCCCACCA TCTGCAAGCA GACCTGTGCC CGGGGACGCT 1120
GTGCCAACAG CTGTGAGAAG GGTGACACCA CCACCTTGTA CAGTCAGGGT GGCCATGGGC ATGACCCCAA GTCTGGCTTC 1200
CGTATCTATT TCTGCCAAAT CCCCTGCCTG AATGGTGGCC GCTGCATCGG CCGGGACGAG TGCTGGTGTC CAGCCAACTC 1280
CACAGGAAAG TTCTGCCATC TGCCTGTCCC GCAGCCAGAC AGGGAACCTG CAGGGCGAGG TTCCCGGCAC AGAACCCTGC 1360
TGGAAGGTCC CCTGAAGCAA TCCACCTTCA CGCTGCCTCT CTCTAACCAG CTCGCCTCTG TGAACCCCTC GCTGGTGAAG 1440
GTGCAAATTC ATCACCCGCC TGAGGCCTCT GTGCAGATTC ACCAGGTGGC CCGGGTCCGG GGTGAGCTGG ACCCCGTGCT 1520
GGAGGACAAC AGTGTGGAGA CCAGAGCCTC TCATCGCCCC CACGGCAACC TAGGCCACAG CCCCTGGGCC AGCAACAGCA 1600
TACCCGCTCG GGCCGGAGAG GCCCCTCGGC CACCACCAGT GCTGTCTAGG CATTATGGAC TTCTGGGCCA GTGTTACCTG 1680
AGCACGGTGA ATGGACAGTG TGCTAACCCC CTAGGTAGTC TGACTTCTCA GGAGGACTGC TGTGGCAGTG TGGGGACCTT 1760
CTGGGGGGTG ACCTCCTGTG CTCCCTGCCC ACCCAGACAA GAGGGTCCAG CCTTCCCAGT GATTGAAAAT GGCCAGCTGG 1840
AGTGTCCCCA AGGATACAAG AGACTGAACC TCAGCCACTG CCAAGATATC AATGAGTGCC TGACCCTGGG CCTCTGCAAG 1920
```

FIG. 27-1

GACTCGGAGT GCGTGAACAC CAGGGGCAGC TACCTGTGCA CCTGCAGGCC TGGCCTCATG CTGGATCCGT CAAGGAGCCG 2000
CTGCGTATCG GACAAGGCTG TCTCCATGCA GCAGGGACTA TGCTACCGGT CACTGGGGTC TGGTACCTGC ACCCTGCCTT 2080
TGGTTCATCG GATCACCAAG CAGATATGCT GCTGCAGCCG TGTGGGCAAA GCCTGGGGTA GCACATGTGA ACAGTGTCCC 2160
CTGCCTGGCA CAGAAGCCTT CAGGGAGATC TGCCCTGCTG GCCATGGCTA CACCTACTCG AGCTCAGACA TCCGCCTGTC 2240
TATGAGGAAA GCCGAAGAAG AGGAACTGGC TAGCCCCTTA AGGGAGCAGA CAGAGCAGAG CACTGCACCC CCACCTGGGC 2320
AAGCAGAGAG GCAACCACTC CGGGCAGCCA CCGCCACCTG GATTGAGGCT GAGACCCTCC CTGACAAAGG TGACTCTCGG 2400
GCTGTTCAGA TCACAACCAG TGCTCCCCAC CTACCTGCCC GGGTACCAGG GGATGCCACT GGAAGACCAG CACCATCCTT 2480
GCCTGGACAG GGCATTCCAG AGAGTCCAGC AGAAGAGCAA GTGATTCCCT CCAGTGATGT CTTGGTGACA CACAGCCCCC 2560
CAGACTTTGA TCCATGTTTT GCTGGAGCCT CCAACATCTG TGGCCCTGGG ACCTGTGTGA GCCTCCCAAA TGGATACAGA 2640
TGTGTCTGCA GCCCTGGCTA CCAGCTACAC CCCAGCCAAG ACTACTGTAC TGATGACAAC GAGTGTATGA GGAACCCCTG 2720
TGAAGGAAGA GGGCGCTGTG TCAACAGTGT GGGCTCCTAC TCCTGCCTCT GCTATCCTGG CTACACACTA GTCACCCTCG 2800
GAGACACACA GGAGTGCCAA GATATCGATG AGTGTGAGCA GCCCGGGGTG TGCAGTGGTG GGCGATGCAG CAACACGGAG 2880
GGCTCGTACC ACTGCGAGTG TGATCGGGGC TACATCATGG TCAGGAAAGG ACACTGTCAA GATATCAACG AATGCCGTCA 2960
CCCTGGTACC TGCCCTGATG GGAGATGCGT CAACTCCCCT GGCTCCTACA CTTGTCTGGC CTGTGAGGAG GGCTATGTAG 3040
GCCAGAGTGG GAGCTGTGTA GATGTCAATG AGTGTCTGAC CCCTGGGATA TGTACCCATG GAAGGTGCAT CAACATGGAA 3120
GGCTCCTTTA GATGCTCCTG TGAGCCGGGC TATGAGGTCA CCCCAGACAA GAAGGGCTGC CGAGATGTGG ACGAGTGTGC 3200
CAGCCGAGCC TCGTGCCCCA CGGGCCTCTG CCTCAACACG GAGGGCTCCT TCACCTGCTC AGCCTGTCAG AGCGGGTACT 3280
GGGTGAACGA AGATGGCACT GCCTGTGAAG ACTTGGATGA ATGTGCCTTC CCTGGAGTCT GCCCCACAGG CGTCTGCACC 3360
AATACTGTAG GCTCCTTCTC CTGCAAGGAC TGTGACCAGG GCTACCGGCC CAACCCCCTG GGCAACAGAT GCGAAGATGT 3440
GGATGAGTGT GAAGGTCCCC AAAGCAGCTG CCGGGGAGGC GAATGCAAGA ACACAGAAGG TTCCTACCAA TGCCTCTGTC 3520
ACCAGGGCTT CCAGCTGGTC AATGGCACCA TGTGTGAGGA CGTGAATGAG TGTGTTGGGG AAGAGCATTG TGCTCCTCAC 3600
GGCGAGTGCC TCAACAGCCT GGGCTCCTTC TTCTGCCTCT GTGCACCCGG CTTTGCTAGT GCTGAGGGGG GCACCAGATG 3680
CCAGGATGTT GATGAATGTG CAGCCACAGA CCCGTGTCCG GGAGGACACT GTGTCAACAC AGAGGGCTCC TTCAGCTGTC 3760
TGTGTGAGAC TGCTTCCTTC CAGCCCTCCC CAGACAGCGG AGAATGTTTG GATATTGATG AGTGTGAGGA CCGTGAAGAC 3840

FIG. 27-2

CCGGTGTGCG GAGCCTGGAG GTGTGAGAAC AGTCCTGGTT CCTACCGCTG CATCCTGGAC TGCCAGCCTG GATTCTATGT 3920
GGCGCCAAAT GGAGACTGCA TTGACATAGA TGAATGTGCC AATGACACTG TGTGTGGGAA CCATGGCTTC TGTGACAACA 4000
CGGACGGCTC CTTCCGCTGC CTGTGTGACC AGGGCTTCGA GACCTCACCA TCAGGCTGGG AGTGTGTTGA TGTGAACGAG 4080
TGTGAGCTCA TGATGGCAGT GTGTGGGGAT GCGCTCTGTG AGAACGTGGA AGGCTCCTTC CTGTGCCTTT GCGCCAGTGA 4160
CCTTGAGGAG TACGACGCAG AAGAAGGACA CTGCCGTCCT CGGGTGGCTG GAGCTCAGAG AATCCCAGAG GTCCGGACAG 4240
AGGACCAGGC TCCAAGCCTT ATCCGCATGG AATGCTACTC TGAACACAAT GGTGGTCCTC CCTGCTCTCA AATCCTGGGC 4320
CAGAACTCCA CACAGGCCGA GTGCTGCTGC ACTCAGGGTG CCAGATGGGG AAAGGCCTGT GCGCCCTGCC CATCTGAGGA 4400
CTCAGTTGAA TTCAGTCAGC TCTGCCCCAG TGGTCAAGGT TACATCCCAG TGGAAGGAGC CTGGACATTT GGACAAACCA 4480
TGTATACAGA TGCCGATGAA TGTGTACTGT TTGGGCCTGC TCTCTGCCAG AATGGCCGAT GCTCAAACAT AGTGCCTGGC 4560
TACATTTGCC TGTGCAACCC TGGCTACCAC TATGATGCCT CCAGCAGGAA GTGCCAGGAT CACAACGAAT GCCAGGACTT 4640
GGCCTGTGAG AACGGTGAGT GTGTGAACCA AGAAGGCTCC TTCCATTGCC TCTGCAATCC CCCCCTCACC CTAGACCTCA 4720
GTGGGCAGCG CTGTGTGAAC ACGACCAGCA GCACGGAGGA CTTCCCTGAC CATGACATCC ACATGGACAT CTGCTGGAAA 4800
AAAGTCACCA ATGATGTGTG CAGCCAGCCC TTGCGTGGGC ACCATACCAC CTATACAGAA TGCTGCTGCC AAGATGGGGA 4880
GGCCTGGAGC CAGCAATGCG CTCTGTGCCC GCCCAGGAGC TCTGAGGTCT ACGCTCAGCT GTGCAACGTG GCTCGGATTG 4960
AGGCAGAGCG CGGAGCAGGG ATCCACTTCC GGCCAGGCTA TGAGTATGGC CCTGGCCTGG ACGATCTGCC TGAAAACCTC 5040
TACGGCCCAG ATGGGGCTCC CTTCTATAAC TACCTAGGCC CCGAGGACAC TGCCCCTGAG CCTCCCTTCT CCAACCCAGC 5120
CAGCCAGCCG GGAGACAACA CACCTGTCCT TGAGCCTCCT CTGCAGCCCT CTGAACTTCA GCCTCACTAT CTAGCCAGCC 5200
ACTCAGAACC CCCTGCCTCC TTCGAAGGCC TTCAGGCTGA GGAATGTGGC ATCCTGAATG GCTGTGAGAA TGGCCGCTGC 5280
GTGCGTGTGC GGGAGGGCTA CACTTGCGAC TGCTTTGAGG GCTTCCAGCT GGATGCGCCC ACATTGGCCT GTGTGGATGT 5360
GAACGAGTGT GAAGACTTGA ACGGGCCTGC ACGACTCTGT GCACACGGTC ACTGTGAGAA CACAGAGGGT TCCTATCGCT 5440
GCCACTGTTC GCCAGGTTAC GTGGCAGAGC CAGGCCCCCC ACACTGTGCG GCCAAGGAGT AG 5502

FIG. 27-3

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MESTSPRGLRCPQLCSHSGAMRAPTTARCSGCIQRVRWRGFLPLVLAVLMGTSHAQRDSIGRYEPASRDANRLWHPVGSHPAAAAAKVYS    90
LFREPDAPVPGLSPSEWNQPAQGNPGWLAEAEARRPPRTQQLRRVQPPVQTRRSHPRGQQQIAARAAPSVARLETPQRPAAARRGRLTGR   180
NVCGGQCCPGWTTSNSTNHCIKPVCQPPCQNRGSCSRPQVCICRSGFRGARCEEVIPEEEFDPQNARPVPRRSVERAPGPHRSSEARGSL   270
VTRIQPLVPPPSPPPSRRLSQPWPLQQHSGPSRTVRRYPATGANGQLMSNALPSGLELRDSSPQAAHVNHLSPPWGLNLTEKIKKIKVVF   360
TPTICKQTCARGRCANSCEKGDTTTLYSQGGHGHDPKSGFRIYFCQIPCLNGGRCIGRDECWCPANSTGKFCHLPVPQPDREPAGRGSRH   450
RTLLEGPLKQSTFTLPLSNQLASVNPSLVKVQIHHPPEASVQIHQVARVRGELDPVLEDNSVETRASHRPHGNLGHSPWASNSIPARAGE   540
APRPPPVLSRHYGLLGQCYLSTVNGQCANPLGSLTSQEDCCGSVGTFWGVTSCAPCPPRQEGPAFPVIENGQLECPQGYKRLNLSHCQDI   630
NECLTLGLCKDSECVNTRGSYLCTCRPGLMLDPSRSRCVSDKAVSMQQGLCYRSLGSGTCTLPLVHRITKQICCCSRVGKAWGSTCEQCP   720
LPGTEAFREICPAGHGYTYSSSDIRLSMRKAEEEELASPLREQTEQSTAPPPGQAERQPLRAATATWIEAETLPDKGDSRAVQITTSAPH   810
LPARVPGDATGRPAPSLPGQGIPESPAEEQVIPSSDVLVTHSPPDFDPCFAGASNICGPGTCVSLPNGYRCVCSPGYQLHPSQDYCTDDN   900
ECMRNPCEGRGRCVNSVGSYSCLCYPGYTLVTLGDTQECQDIDECEQPGVCSGGRCSNTEGSYHCECDRGYIMVRKGHCQDINECRHPGT   990
CPDGRCVNSPGSYTCLACEEGYVGQSGSCVDVNECLTPGICTHGRCINMEGSFRCSCEPGYEVTPDKKGCRDVDECASRASCPTGLCLNT  1080
EGSFTCSACQSGYWVNEDGTACEDLDECAFPGVCPTGVCTNTVGSFSCKDCDQGYRPNPLGNRCEDVDECEGPQSSCRGGECKNTEGSYQ  1170
CLCHQGFQLVNGTMCEDVNECVGEEHCAPHGECLNSLGSFFCLCAPGFASAEGGTRCQDVDECAATDPCPGGHCVNTEGSFSCLCETASF  1260
QPSPDSGECLDIDECEDREDPVCGAWRCENSPGSYRCILDCQPGFYVAPNGDCIDIDECANDTVCGNHGFCDNTDGSFRCLCDQGFETSP  1350
SGWECVDVNECELMMAVCGDALCENVEGSFLCLCASDLEEYDAEEGHCRPRVAGAQRIPEVRTEDQAPSLIRMECYSEHNGGPPCSQILG  1440
QNSTQAECCCTQGARWGKACAPCPSEDSVEFSQLCPSGQGYIPVEGAWTFGQTMYTDADECVLFGPALCQNGRCSNIVPGYICLCNPGYH  1530
YDASSRKCQDHNECQDLACENGECVNQEGSFHCLCNPPLTLDLSGQRCVNTTSSTEDFPDHDIHMDICWKKVTNDVCSQPLRGHHTTYTE  1620
CCCQDGEAWSQQCALCPPRSSEVYAQLCNVARIEAERGAGIHFRPGYEYGPGLDDLPENLYGPDGAPFYNYLGPEDTAPEPPFSNPASQP  1710
GDNTPVLEPPLQPSELQPHYLASHSEPPASFEGLQAEECGILNGCENGRCVRVREGYTCDCFEGFQLDAPTLACVDVNECEDLNGPARLC  1800
AHGHCENTEGSYRCHCSPGYVAEPGPPHCAAKE                                                          1833
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FIG. 28